Reactive Oxygen Species and Organellar Signaling

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Highlight

Reactive oxygen species (ROS) in different organelles (chloroplasts, mitochondria, and peroxisomes) trigger distinct, yet potentially intersecting, organellar signaling pathways that mediate operational readjustments of the respective organelle during environmental stresses.

Abstract

The evolution of photosynthesis and its associated metabolic pathways has been crucial to the successful establishment of plants, but has also challenged plant cells in the form of reactive oxygen species (ROS) production. Intriguingly, multiple forms of ROS are generated in virtually every plant cell compartment through diverse pathways. As a result, a sophisticated network of ROS detoxification and signaling that is simultaneously tailored to individual organelles and safeguards the entire cell is necessary. Here we take an organelle-centric view on the principal sources and sinks of ROS across the plant cell and give insights into the ROS-induced organelle-to-nucleus retrograde signaling pathways needed for operational readjustments during environmental stresses.

Key words

Chloroplasts, metabolism, mitochondria, peroxisomes, photorespiration, photosynthesis, reactive oxygen species (ROS), retrograde signaling

Abbreviations

$^1O_2$, Singlet oxygen; 2PG, 2-phosphoglycolate; 3PGA, 3-phosphoglycerate; APX, ascorbate peroxidase; CO_2, carbon dioxide; ETC, electron transport chain; GPX, glutathione peroxidase; H_2O_2, hydrogen peroxide; MecPP, methylerythritol cyclodiphosphate; O_2, oxygen; $O_2^{\cdot-}$, superoxide anions; $OH^{\cdot}$, hydroxyl radical; PAP, 3'-phosphoadenosine 5'-phosphate; PRX, peroxiredoxins; PSII, photosystem II; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SA, salicylic acid; SODs, superoxide dismutases; Trx, thioredoxin; β-CC, β-cyclocitral.

Introduction

Oxygenic photosynthesis converts solar energy into biochemical energy by splitting water and releasing oxygen (O_2). This process gradually modified the ancient anaerobic atmosphere [mainly composed of methane, nitrogen, and carbon dioxide (CO_2), with oxygen levels below 0.1%] to the present more oxygen-enriched earth's atmosphere (Holland, 2006; Luo *et al.*, 2016), driving the evolution of aerobic lifestyles. The enrichment in atmospheric oxygen enabled the establishment of the ozone layer, which protects living organisms from harmful UV-radiation. Moreover, the use of oxygen as the final electron acceptor of electron transport chains (ETC) in cellular respiration boosted cellular energy production, yielding up to 15 fold more ATP from carbon sources, such as glucose (Halliwell, 2006).

The increased oxygen abundance within plant cells also came with a trade-off, namely the enhanced production of partially reduced or excited forms of oxygen, collectively known as reactive oxygen species (ROS) [reviewed by Noctor and Foyer (2016); Waszczak *et al.* (2018)]. ROS are highly reactive and can oxidize various biological molecules, such as DNA, lipid, and proteins, causing cellular damage (Van Breusegem and Dat, 2006). Changes in environmental conditions, such as water availability, light intensity, temperature, and pathogen presence, trigger a surge in ROS production in organelles, such as chloroplasts and mitochondria, resulting in oxidative stress in plants. However, plants can also utilize the reactive properties of ROS to their benefit. For example, in a developmental context, by strategically increasing ROS concentrations at the apoplast to loosen the existing rigid cell wall for cell expansion during growth (Kärkönen and Kuchitsu, 2015) or to kill invading pathogens by launching a NADPH oxidase-dependent oxidative burst (Kadota *et al.*, 2015). A highly sophisticated regulatory network exists in plant cells to sense, respond to, detoxify, and utilize ROS. Intriguingly, recent discoveries highlight a prominent influence of organelle compartmentation on this ROS network. Therefore, this review takes on an organelle-centric view to examine the nature and mode of cellular acclimation to ROS.

ROS metabolism in plant cells

Unique and common aspects of ROS

Different ROS have distinct chemical reactivity characteristics that determine their half-lives and migration distance at the cellular level. For a useful summary of ROS types and characteristics, we refer the reader to Tripathy and Oelmüller (2012), Das and Roychoudhury (2014), and Mittler (2017). The hydroxyl radical ($OH^{\cdot}$) is the most reactive of all ROS and, hence, the shortest lived (half-life of 1 ns). Singlet oxygen ($^1O_2$) and superoxide anions ($O_2^{\cdot -}$) are relatively longer-lived ROS (half-life of 1-4 µs) and moderately reactive, although $O_2^{\cdot -}$ is rapidly converted to H_2O_2 either by superoxide dismutases (SODs) or via nonenzymatic dismutation (Bielski and Allen, 1977). Consequently $O_2^{\cdot -}$ has a relatively shorter migration distance range than $^1O_2$. H_2O_2 is considered the most stable ROS with the longest lifetime and can migrate through the cell (in µm range).

With the exception of $^1O_2$, which is mostly generated at photosystem II (PSII) in thylakoid membranes of chloroplasts, the other three ROS are found across multiple subcellular compartments. Both $O_2^{\cdot -}$ and H_2O_2 are produced in mitochondria, chloroplasts, peroxisomes, and the apoplast. $OH^{\cdot}$ is also produced in all of these compartments, because it derives from H_2O_2 in an Fe^{2+}-dependent reaction. Similarly, the removal of $O_2^{\cdot -}$, H_2O_2, and $OH^{\cdot}$ share similar mechanisms across different organelles. For example, each of the subcellular compartments contains at least one member of the SOD family for $O_2^{\cdot -}$ detoxification (Alscher *et al.*, 2002). H_2O_2 homeostasis across the different organelles is mainly determined by a core set of ubiquitous redox couples, including ascorbate (DHA/Asc), NAD(P)+/NAD(P)H, and thiol (-SH, sulfhydryl group)-containing couples, such as thioredoxin (Trx), ferredoxin (Fd), and glutathione (GSH/GSSG). The reduction of these redox couples typically passes through a cycling process of thiol relays with NAD(P)H feeding the cycle with electrons (Mittler *et al.*, 2004). Additionally, H_2O_2 can be detoxified by catalases (Mhamdi *et al.*, 2010). The most reactive $OH^{\cdot}$ is probably scavenged nonenzymatically by nearby sugars and proteins to form less reactive oxidized sugar and protein radicals (Matros *et al.*, 2015; Kumar *et al.*, 2019).

Metabolism is a major ROS source in plants

Energy metabolism in plant cells not only links chloroplasts, mitochondria, and peroxisomes together in the channeling and conversion of carbon-containing compounds, but also generates ROS as by-products in all three organelles (Fig. 1), although in comparison to mammalian cells, chloroplasts and peroxisomes are considered the major ROS sources in illuminated photosynthetic cells rather than mitochondria (Noctor *et al.*, 2002). The high rate

of electron and energy transfers within the plant energy organelles are crucial drivers that facilitate ROS production both at the thylakoid membrane of chloroplasts during photosynthetic light reactions and at the mitochondrial inner membrane ETC during cellular respiration (see below).

The second driver for ROS overproduction is the key player in carbon-fixing photosynthesis, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The evolution of Rubisco as a CO_2-carboxylating enzyme preceded the aforementioned atmospheric oxygenation that occurred over a relatively shorter timescale (Iñiguez *et al.*, 2020). Consequently Rubisco has poor CO_2/O_2 discrimination and frequently incorporates O_2 rather than CO_2 to form 2-phosphoglycolate (2PG) instead of 3-phosphoglycerate (3PGA) in the Calvin-Benson cycle. 2PG can negatively impact on carbon fixation by (i) draining the carbon substrate from the Calvin-Benson cycle, when not recycled and (ii) more importantly, inhibiting enzyme activities, including those of enzymes that function downstream of Rubisco in the Calvin-Benson cycle, such as triose-phosphate isomerase and sedoheptulose 1,7-bisphosphate phosphatase (Norman and Colman, 1991; Flügel *et al.*, 2017). To minimize these impacts, 2PG is rapidly degraded and ultimately converted back to 3PGA via photorespiration, a light-induced biochemical process that consumes O_2 and releases CO_2 (Hagemann *et al.*, 2016). Photorespiration spans across multiple cellular compartments (chloroplasts, peroxisomes, mitochondria, and cytosol) and ROS in the form of hydrogen peroxide (H_2O_2) is produced within the peroxisomes via the action of glycolate oxidase (Fig. 1). In cells of C3 plants, photorespiration is estimated to account for approximately 70% of the H_2O_2 production (Noctor *et al.*, 2002). By contrast, in algae, the oxidation of glycolate is catalyzed either by a glycolate oxidase, as in plants, or by a glycolate dehydrogenase, which does not generate H_2O_2. The green microalga *Chlamydomonas reinhardtii* encodes both enzymes in its genome. Glycolate oxidation is performed by the mitochondria-localized glycolate dehydrogenase while the so-called glycolate oxidase is rather a lactate oxidase (Kern *et al.*, 2020).

Chloroplastic ROS metabolism

When light intensity exceeds the photosynthetic capacity, photosynthetic organisms trigger different mechanisms to protect their photosynthetic ETC by minimizing production, or increasing the scavenging, of ROS (Müller *et al.*, 2001; Murchie and Ruban, 2020). One prominent set of short-term adaptations, named ‘nonphotochemical quenching’ (NPQ), is

associated with PSII (Horton *et al.*, 1996) and include processes, such as (i) state transitions (qT), (ii) high energy-dependent quenching (qE), and (iii) photoinhibition (qI).

State transitions are important for the optimization of the energy distribution between PSI and PSII (Ruban and Johnson, 2009; Stirbet *et al.*, 2020). They are regulated by a kinase [state transition 7 (STT7) in *Chlamydomonas* and STN7 in higher plants (Rochaix, 2007)] of which the activation requires binding of plastoquinol to the cytochrome b_6f complex (Wollman, 2001). This kinase phosphorylates light-harvesting complex II (LHCII) and the phosphorylated LHCII disconnects from PSII and becomes a PSI antenna (state 2). When the plastoquinone pool is oxidized, the kinase is inactivated, the LHCII is dephosphorylated by a phosphatase (PPHI/TAP38), and moves back to PSII (state 1) [reviewed by (Stirbet *et al.*, 2020)]. Interestingly, qT is very pronounced in cyanobacteria and algae, but is a weaker response in higher plants where it is not associated with a change in gene expression (Ruban and Johnson, 2009).

High energy-dependent quenching (qE) corresponds to the deactivation of the PSII antenna by heat and is induced by three factors: (i) an acidification of the pH in the lumen, (ii) the xanthophyll cycle that catalyzes the conversion of violaxanthin into antheraxanthin and zeaxanthin, and (iii) a PSII subunit, PsbS (Stirbet *et al.*, 2020). In algae, the qE extent depends on the light-harvesting complex stress-related (LHCSR) proteins (Peers *et al.*, 2009) and to a lesser extent on PsbS (Correa-Galvis *et al.*, 2016).

Photoinhibition (qI) occurs when the other mechanisms cannot alleviate the light stress. It corresponds to damage to D1, the PSII reaction center, produced by singlet oxygen at the acceptor side of PSII or the production of long-lived highly oxidizing P680 at the donor site of PSII (Andersson and Aro, 2001).

It is noteworthy that qT, qE and qI function at different timescales relative to ROS accumulation [reviewed in Dietz (2015)]. Energy quenching via qE is activated within seconds of exposure to higher light intensity and qT functions over a period of minutes thereafter; whereas qI occurs on a timeframe of hours. Chloroplastic ROS accumulation has been mostly documented to occur within minutes-hours post-exposure to high light; thus qE and qT most likely prevent ROS accumulation while qI serves to alleviate damage by ROS overaccumulation. This is exemplified by the higher $^1O_2$ accumulation in high light stressed *npq1*, deficient in the light-dependent violaxanthin to zeaxanthin interconversion component of qE, compared to wild type (Dall'Osto *et al.*, 2012).

As mentioned above, singlet oxygen is commonly produced in the PSII center, when excited chlorophyll molecules react in a triplet state (^{3}Chl) with oxygen (Fischer *et al.*, 2013)

rather than when its excess energy is quenched by nearby pigments, such as carotenoids (Fig. 1). Singlet oxygen can also be produced in the presence of biosynthetic intermediates of chlorophyll (protochlorophyllide or protoporphyrin IX), which are photosensitizers, upon light exposure (Wang *et al.*, 2016). Production of $^1O_2$ from chlorophyll intermediates can occur either on thylakoid membranes or in the prolamellar body of proplastids and etioplasts of developing seedlings when first exposed to light after germination in soil (Solymosi and Schoefs, 2010). These chlorophyll intermediates are normally kept at low levels under the tight regulation of chlorophyll production to minimize $^1O_2$ production in wild type plants. In *Arabidopsis thaliana*, the *flu* mutant deficient in rapid light-responsive conversion of protochlorophyllide to avoid $^1O_2$ accumulation has served as a valuable tool to elucidate $^1O_2$ detoxification and signaling (discussed later). For example, usage of *flu* as a conditional tool for light-induced $^1O_2$ accumulation showed that one of the first sites of damage is the grana margin, which is protected by the SAFEGUARD1 protein (Wang *et al.*, 2020a). Singlet oxygen is also quenched by carotenoids, tocopherols, and plastoquinones at thylakoid membranes close to its production site; where the excess energy of $^1O_2$ is normally absorbed by these molecules to avoid lipid peroxidation and photoinhibition (Krieger-Liszkay and Trebst, 2006; Ramel *et al.*, 2012). Ubiquinol, ascorbate, and glutathione are also known to contribute to $^1O_2$ quenching (Laloi and Havaux, 2015).

Within the chloroplastic ETC, electrons can "leak" toward oxygen, thereby generating $O_2^{\cdot -}$ (Roach and Krieger-Liszkay, 2014). Most of this electron leakage occurs at the level of PSI, where ferredoxin conducts electrons toward O_2 instead of $NADP^+$, a process known as the Mehler reaction. This electron leak could be beneficial during CO_2-limiting conditions, particularly during environmental stresses, because it provides an alternative sink to sustain electron flow of photosynthesis (Ort and Baker, 2002; Sun *et al.*, 2020). In algae and cyanobacteria, the Mehler reaction functions as an important electron sink involving 10 to 50% of the photosynthetic electron flux (Badger *et al.*, 2000). However, in higher plants, especially in angiosperms, the Mehler reaction rate is limited from negligibly small to less than 10% of the linear electron flow (Shirao *et al.*, 2013; Mullineaux *et al.*, 2018). $O_2^{\cdot -}$ is rapidly neutralized by SODs to the less toxic species H_2O_2 (Fig. 1).

In the chloroplasts, iron (Fe)SODs and the membrane-bound-copper/zinc (Cu/Zn)SODs are responsible for the conversion of $O_2^{\cdot -}$ to H_2O_2 (Alscher *et al.*, 2002; Pilon *et al.*, 2011), which is subsequently reduced to water by ascorbate and thiol peroxidases. Of the nine ascorbate peroxidases (APXs) in *Arabidopsis* that use ascorbate as an electron donor to reduce H_2O_2 to H_2O, two localize to the chloroplasts [see review by Maruta *et al.* (2016) for detailed

information on chloroplastic APXs]. The thylakoid (t)APX isoform serves as a first-line detoxification system to remove H_2O_2 produced at PSI (Fig. 1). In addition, H_2O_2 diffusing throughout the stroma is scavenged by stromal (s)APX (Asada, 2000). Interestingly, although transgenic overexpression of these chloroplastic APXs promote stress resistance in plants (Yabuta *et al.*, 2002), loss-of-function mutants do not display any severe phenotypes under laboratory settings (Giacomelli *et al.*, 2007; Kangasjärvi *et al.*, 2008; Maruta *et al.*, 2010; Caverzan *et al.*, 2014), highlighting the several layers of partially redundant ROS detoxification mechanisms in chloroplasts. For example, in addition to the glutathione-ascorbate cycle (Foyer and Halliwell, 1976; Asada, 2006), thiol peroxidases assist in the detoxification of chloroplastic H_2O_2. The chloroplasts also possess multiple thioredoxin types, including TRXm, TRXf, TRXx, TRXy, TRXz, and NADPH-dependent TRX reductase C (NTRC), several of which are essential for ROS metabolism (Buchanan, 2016; Nikkanen and Rintamäki, 2019).

Mitochondrial ROS metabolism

Similar to chloroplasts, the mitochondrial (m)ETC generates ROS. During respiration, 1% of the oxygen consumption has been estimated to generate H_2O_2 in plants (Puntarulo *et al.*, 1988). Superoxide radicals can be formed at complex I and complex III of mETC, when oxygen is only reduced by a single electron (Fig. 1). Superoxides are produced in the flavoprotein region of NADH dehydrogenase (complex 1) in the mitochondrial matrix (Møller, 2001). At the complex III level, fully reduced ubiquinone (QH_2) passes electrons to cytochrome *c*, leaving an unstable ubisemiquinone radical that favors O_2 as an electron acceptor, thus forming $O_2^{\cdot-}$ radicals in both the matrix and in the intermembrane space. Enzymes in the matrix could also contribute to superoxide and H_2O_2 production, either at their flavin site (such as 2-oxoacid dehydrogenase complexes) or at their quinone-binding site (such as mitochondrial 3-phosphate dehydrogenase) [reviewed in Larosa and Remacle (2018)]. In the matrix, $O_2^{\cdot-}$ is rapidly neutralized to H_2O_2 by MnSOD and, hence, $O_2^{\cdot-}$ levels in the matrix are probably relatively low. However, it is unclear how $O_2^{\cdot-}$ is detoxified within the intermembrane space in plants. In yeast (Field *et al.*, 2003) and mammals (Iñarrea *et al.*, 2005), Cu/ZnSOD has been shown to localize to the intermembrane space to detoxify $O_2^{\cdot-}$. Given the evolutionarily conserved antioxidant machinery in mitochondria, a Cu/ZnSOD might play similar role in plants as well. In addition, $O_2^{\cdot-}$ might be scavenged by ascorbate (Halliwell and Foyer, 1976; Smirnoff, 2018), which is synthesized in the intermembrane

space (Bartoli *et al.*, 2000). Both $O_2^{\cdot-}$ and its dismutation product H_2O_2 have been proposed to diffuse out to the cytosol, where they are subsequently detoxified or act as signaling molecules by altering local cytosolic redox states (Waszczak *et al.*, 2018). Besides the main mETC components, other enzymes also feed electrons to the mETC. Galactono-γ-lactone dehydrogenase (GLDH), in addition to its role in the structural regulation of the mETC complex I (Schimmeyer *et al.*, 2016; Soufari *et al.*, 2020), also catalyzes the last ascorbate biosynthesis step that provides an electron to cytochrome *c* (Bartoli *et al.*, 2000), as such contributing to an overreduction of the ubiquinone pool and, hence, increasing the mitochondrial ROS levels.

Mitochondrial H_2O_2 is neutralized to H_2O by thiol and APXs, as well as by associated cycles similar to those in chloroplasts (Fig. 1). In fact, mitochondria and chloroplasts share most of the H_2O_2-scavenging enzymes, including APX, glutathione peroxidase (GPX), peroxiredoxin (PRX) II, monodehydroascorbate reductase (MDHAR), and glutathione reductase (Creissen *et al.*, 1995; Chew *et al.*, 2003; Rouhier *et al.*, 2005; Navrot *et al.*, 2006). In *Arabidopsis*, the stromal APX is dually targeted to chloroplasts and to the inner membrane facing the intermembrane space of mitochondria (Chew *et al.*, 2003), suggesting that H_2O_2 as well as the APX activity-formed monodehydroascorbate must migrate through the membrane, because the other enzymes of the ascorbate-glutathione cycle are located in the matrix (Edwards *et al.*, 1990; Creissen *et al.*, 1995; Jimenez *et al.*, 1997). In tomato (*Solanum lycopersicum*), however, APX isoforms have been detected both in the intermembrane space as well as in the matrix (Mittova *et al.*, 2004). Although the exact suborganellar localization has not completely been elucidated in plants yet, it is evident that an ascorbate-glutathione cycle is present in the mitochondria (Møller, 2001; Chew *et al.*, 2003). In contrast to chloroplasts, only GPX and PRXII, but not PRXQ nor 2-Cys PRX, occur in mitochondria. Mitochondria also possess an NADP/Trx system which is governed by Trx-o1, Trx-o2, and NTRA, the latter obtaining electrons from NADPH (Laloi *et al.*, 2001; Meyer *et al.*, 2012).

Additional pathways are found in the mitochondria for the indirect regulation of ROS levels. The mETC in plants is highly branched and extra “alternative oxidoreductases” contribute to the electron flow, without producing energy (Fig. 1) (Møller, 2001; Rasmusson *et al.*, 2008; Schertl and Braun, 2014). These alternative paths for electron transport allow plants to adjust their metabolism as required (Rasmusson *et al.*, 2008). Type II NAD(P)H dehydrogenases (NDs) introduce electrons to the ubiquinone pool, thereby by-passing complex I, and are located both on the inner (ND_{in}) and external (ND_{ex}) sides of the inner membrane. Because NDs have a relatively low affinity for NAD(P)H compared to complex I,

they are believed to act only during NADH excess (Møller *et al.*, 1993). However, because they can oxidize NAD(P)H originating from either the mitochondrial matrix or from the cytosol (diffused to the intermembrane space), they play a role in redox homeostasis and signaling, particularly during stress (Rasmusson *et al.*, 2008; Podgórska *et al.*, 2018). In *Chlamydomonas*, the ND_{in} has been characterized: no phenotype is detected when it is inactivated, but when the mutation is combined with a complex I mutation, the phenotype is much more severe than that of a single complex I mutant, suggesting that Nd_{in} also contributes to the NADH recycling for redox balance in the absence of stress conditions (Lecler *et al.*, 2012). Another alternative path, the alternative oxidase (AOX), bypasses complex III and VI by transferring electrons from the ubiquinone pool to O_2, thus preventing overreduction of the ubiquinone pool and ROS production (Umbach *et al.*, 2005). Mutant studies have demonstrated that AOX inhibition of ROS formation is especially important during environmental stress, even when the mETC might not be the primary target of the stress (Umbach *et al.*, 2005; Giraud *et al.*, 2008; Wang *et al.*, 2011; Yoshida *et al.*, 2011; Vishwakarma *et al.*, 2015). The AOX pathway helps to maintain photorespiration in mitochondria by ensuring continuous consumption of NADH generated from the conversion of glycine to serine by complex I, thus, contributing to PSII photoprotection in plants (Zhang *et al.*, 2017), and is beneficial in maintaining both mitochondrial and chloroplastic functions during drought stress (Dahal and Vanlerberghe, 2017).

Peroxisomal ROS metabolism

Peroxisomes house several metabolic processes, which directly produce ROS, among which the main is the peroxisomal module of photorespiration (Fig. 1). As mentioned above, photorespiration is the process that converts 2PG, produced by the oxygenase activity of Rubisco, to 3PGA, the product of the carboxylase activity of Rubisco. This process extends over three organelles via multiple intermediate compounds, with the first step in the peroxisomes that generates H_2O_2 through the glycolate oxidase (GOX) activity. Under nonstressed conditions, approximately 25% of the Rubisco activity results in photorespiration and thereby H_2O_2 production, but highly increases under CO_2-limiting conditions, for example, after stomata closure during stress. Mutants with perturbed photorespiratory pathways display accelerated photoinhibition phenotypes under excess light conditions,

suggesting an important role for photorespiration in preventing photoinhibition under CO_2-limiting conditions (Wingler *et al.*, 2000; Takahashi and Badger, 2011).

Besides GOXs, other enzymes also catalyze reactions that result in H_2O_2 formation in peroxisomes (Fig. 1). In the first β-oxidation step that degrades fatty acids into acetyl-CoA, H_2O_2 is generated through Acyl-CoA oxidases. In sulfur metabolism, the conversion of sulfite to sulfate by sulfite oxidase also results in the production of H_2O_2. Normally, H_2O_2 is rapidly converted to water by catalase (see below), but under conditions of high sulfite levels, catalase is inhibited and the produced H_2O_2 assists in the sulfite detoxification by oxidizing sulfite to sulfate (Hänsch *et al.*, 2006). Two purine metabolism enzymes, xanthine oxidoreductase (XOD) and urate oxidase, are additional ROS sources. XOD can function as either a xanthine oxidase or as a xanthine dehydrogenase, producing either $O_2^{\cdot-}$ or H_2O_2, respectively. However, until now, the presence of this enzyme in plant peroxisomes has only been confirmed in watermelon (*Citrullus lanatus*), castor bean (*Ricinus communis*), and pea (*Pisum sativum*) (Sandalio *et al.*, 1988; del Río and Donaldson, 1995; Corpas *et al.*, 2008). In other plants, such as *Arabidopsis*, only a cytosolic xanthine dehydrogenase isoform has been described (Corpas *et al.*, 2008; Zarepour *et al.*, 2010). Polyamine catabolism by FAD-dependent polyamine oxidases (PAOs) and copper-containing amine oxidases CuAOs also contributes to H_2O_2 production in peroxisomes, the cytosol, and apoplast [see reviews by Corpas *et al.* (2019); Wang *et al.* (2019)].

In pea, castor bean, and potato (*Solanum tuberosum*) tuber peroxisomes, a second source of $O_2^{\cdot-}$ is provided by the small ETC (del Río *et al.*, 2002; del Río and López-Huertas, 2016). Although the small ETC is embedded in the peroxisomal membrane, ROS are generated outside the membrane. In pea, three peroxisomal membrane polypeptides (PMPs) are responsible for $O_2^{\cdot-}$ production (López-Huertas *et al.*, 1999). Two PMPs (MPM18 and MPM 32) use NADH as electron donor, whereas the other (PMP29) uses NADPH. Therefore, this small ETC is believed to be involved in the regeneration of NAD^+ and $NADP^+$ to sustain peroxisomal metabolism (del Río *et al.*, 2002). PMP29 displays cytochrome *c* reductase activity and is the main $O_2^{\cdot-}$source. PMP18 and PMP32 are recognized as cytochrome *b* and MDHAR, respectively (López-Huertas *et al.*, 1999).

Peroxisomal H_2O_2 is mainly removed by catalase (Fig. 1), but peroxisomal PRX, APX, and the associated ascorbate-glutathione cycle also assist in the H_2O_2 removal. However, catalase is considered the dominant enzyme in H_2O_2 removal in peroxisomes, based on the severe stress-sensitive phenotypes in catalase-deficient plants (Mhamdi *et al.*, 2010) that are not observed in peroxisomal APX-deficient plants (Narendra *et al.*, 2006). The high

abundance of catalase in peroxisomes and the nonspecific action of reductants, such as ascorbate or glutathione, might explain why catalase is more effective in peroxisomal H_2O_2 detoxification, despite its lower affinity for H_2O_2. For the dismutation of $O_2^{\cdot -}$ to H_2O_2, varying numbers and types of SOD isoforms in peroxisomes are in place (Nyathi and Baker, 2006). For example, whereas peroxisomes of watermelon possess both Cu/ZnSOD and MnSOD, *Arabidopsis* and sunflower (*Helianthus annuus*) peroxisomes possess only a Cu/ZnSOD (Bueno *et al.*, 1995; Corpas *et al.*, 1998; Rodríguez-Serrano *et al.*, 2007; Huang *et al.*, 2012).

Apoplastic ROS production

In contrast to the organellar ROS production, which occurs due to the nature of their resident metabolic pathways, in the apoplast ROS is produced in plants for growth, stomatal closure, and intercellular stress signaling [see reviews by Baxter *et al.* (2014); Podgórska *et al.* (2017); Chapman *et al.* (2019); Chen and Yang (2020)]. NADPH oxidases (respiratory burst oxidase homologs, RBOHs) are embedded in the plasma membrane and they transfer electrons from the cytoplasmic NADPH to the apoplastic oxygen, forming $O_2^{\cdot -}$ (Fig. 1). Of the ten RBOH proteins in *Arabidopsis*, ROS production by RBOHD and RBOHF have been shown to be especially important for different processes in leaves, including rapid systemic signaling in response to environmental stresses (Miller *et al.*, 2009; Zandalinas *et al.*, 2020), plant-pathogen responses (Torres *et al.*, 2002; Morales *et al.*, 2016), and stomatal closure (Sierla *et al.*, 2016). RBOH activity is posttranslationally regulated by several mechanisms including calcium binding at its EF hand motif, N-terminal phosphorylation by a plethora of kinases including receptor-like cytoplasmic kinases such as Botrytis-Induced Kinase 1 (BIK1) (Kadota *et al.*, 2014) and calcium-dependent protein kinases (Dubiella *et al.*, 2013), and C-terminal phosphorylation by plasma-membrane bound receptor-like kinases (Kimura *et al.*, 2020). The superoxide radicals produced by RBOHs are subsequently dismutated to H_2O_2 spontaneously or by an apoplastic SOD. Although the presence of this apoplastic SOD has been proposed based on biochemical assays of apoplast extracts, the genetic identity of this SOD is still unclear (Waszczak *et al.*, 2018).

In addition to their presence in peroxisomes, PAOs are also localized to the apoplast and thus provide another means for H_2O_2 production in this compartment (Fig. 1). They are responsible for the catabolism of polyamines, such as spermidine and spermine, which simultaneously generate H_2O_2. Interestingly, NADPH oxidases and PAOs form a feed-forward loop to increase ROS levels upon stresses (Gémes *et al.*, 2016). Because the apoplast

requires ROS for cell wall loosening and hence growth, the apoplast has evolved to remain in an oxidized state and entails only a limited antioxidant capacity (Müller *et al.*, 2009; Noctor and Foyer, 2016). Indeed, in contrast to other organelles, in which peroxidases play an important role in the H_2O_2 detoxification, peroxidases in the apoplast perform the reverse reaction and are responsible for H_2O_2 and, subsequently $OH^{\cdot}$ formation (Passardi *et al.*, 2005; Daudi *et al.*, 2012). These additional sources of apoplastic ROS are likely to contribute to the same processes that require RBOH-derived ROS, such as the regulation of stomatal closure (Sierla *et al.*, 2016).

H_2O_2 diffusion to the cytosol and nucleus

Cytosolic H_2O_2 originates from the apoplast and the organelles. Although H_2O_2 can diffuse through the membrane, the lipid bilayer forms a poorly permeable barrier (Bienert *et al.*, 2006). Mutant and heterologous expression studies demonstrated that plasma membrane aquaporins can be activated by kinases to assist in the transport of apoplastic H_2O_2 to the cytosol (Grondin *et al.*, 2015; Rodrigues *et al.*, 2017). A similar mechanism has been proposed for chloroplastic H_2O_2 based on chemical inhibition of aquaporins on isolated chloroplasts, although genetic evidence is still lacking (Borisova *et al.*, 2012) (Fig. 1). When H_2O_2 reaches the cytosol, it is rapidly neutralized by APXs and GPXs (Borisova *et al.*, 2012; Gaber *et al.*, 2012), which are subsequently reduced through the ascorbate-glutathione cycle and thioredoxin/Trx reductase cycle (Mittler *et al.*, 2004; Tripathy and Oelmüller, 2012). Because of its efficient antioxidant capacity, the cytosol has often been perceived as a "buffering zone" that limits intracellular H_2O_2 movements. Nevertheless, recent studies using the H_2O_2 fluorescent protein sensor roGFP2-Orp1 show that some stimuli, such as pathogen elicitors and the signaling molecule H_2S, induce significant RBOH-derived H_2O_2 accumulation in the cytosol (Scuffi *et al.*, 2018; Nietzel *et al.*, 2019).

ROS production has also been detected in the nucleus. The first evidence to suggest that ROS are actively produced in the plant nucleus was provided by experiments with isolated nuclei in which increased H_2O_2 levels were measured upon treatment with calcium (Ashtamker *et al.*, 2007). Later, cryptochromes were proposed as a ROS source in plant nuclei (Consentino *et al.*, 2015; Jourdan *et al.*, 2015) because of a differential accumulation of nuclear ROS in overexpression and loss-of-function *Arabidopsis* mutants of cytochrome 1 (CRY1) and CRY2 (El-Esawi *et al.*, 2017). Nonetheless, the physiological relevance or roles of this ROS source remain to be demonstrated in wild type plants and the molecular

mechanisms behind the cryptochrome-mediated photoresponses are still being debated (Arthaut *et al.*, 2017; Wang and Lin, 2020). Whereas no nuclear aquaporin has been described in plants, nuclear H_2O_2 can also accumulate through diffusion, presumably via the large nuclear pores. Nuclear H_2O_2 can originate from either the cytosol or from the organelles directly. H_2O_2 transfer from organelles can be facilitated through the physical association of the organelles to the nucleus (Exposito-Rodriguez *et al.*, 2017). Organelles can also form extensions in the form of liquid tubules that could associate with the nucleus, called stromules (chloroplasts), matrixules (mitochondria), and peroxules (peroxisomes) (Noctor and Foyer, 2016). However, experimental evidence is only available for H_2O_2 transport via stromules into nuclei (Caplan *et al.*, 2015) (Fig. 1).

Control of nuclear redox state is important for plant function. For example, in root cells of glutathione biosynthesis-deficient plants, the more oxidized redox state of their nuclei is accompanied by cell cycle arrest (Schnaubelt *et al.*, 2015). In addition to redox buffers, nuclear H_2O_2 is also enzymatically regulated. Whereas none of the H_2O_2 detoxification enzymes discussed above (e.g. catalase, APX, etc.) are localized to the plant nucleus endogenously (Martins *et al.*, 2018), H_2O_2 in the seed nucleus is neutralized by 1-Cys peroxiredoxins (Pulido *et al.*, 2009), which is recycled via the Trx/Trx reductase cycle. In *Arabidopsis*, four Trx and eight Grx have been assigned a nuclear localization, although they are often associated with the cytosol as well (Delorme-Hinoux *et al.*, 2016). Interestingly, their subcellular location can depend on environmental conditions. For example, GRXS17 resides in the cytosol under standard conditions, but accumulates in the nucleus upon heat treatment (Wu *et al.*, 2012).

ROS metabolism across organelles: some take-home messages

When considering current knowledge on ROS production and processing in the different organelles, some generalised principles are apparent. First, metabolic perturbation leading to electron leakage toward molecular oxygen (both enzymatic and non-enzymatically) represent a shared mechanism for ROS production in chloroplasts, mitochondria and peroxisomes. These organelles coincidentally contribute to the bulk of ROS accumulation in plant cells during abiotic stress. Second, the different organelles utilize similar ROS detoxification strategies entailing ROS-quenching metabolites and multi-gene enzyme families. Intriguingly, organelle-specific differences remain in the types of enzyme(s) and metabolite(s) involved in both the production and detoxification of different ROS. Whether these differences are the

result of specializations due to the unique biochemical environment of each organelles, or are simply outcomes of random gene evolution, would be interesting to explore. Further insights could come from systematically studying the conservation and/or diversification of these organellar ROS metabolism across the evolutionary lineages.

In contrast to the organelles mentioned above, ROS production at the apoplast is initiated and controlled by the cell for stress responses rather than as consequences of metabolic perturbations (Kimura *et al.*, 2020; Zandalinas *et al.*, 2020). This difference between the apoplast and other organelles sets the scene of the dual nature of ROS as both harmful and useful metabolites, depending on their subcellular and metabolic context (Mittler, 2017). Collectively, these observations hint at the complex nature of evolution and specialization of ROS metabolism in plant cells.

ROS-induced organellar signaling

ROS accumulation is tuned to environmental stimuli that induce large-scale cellular responses, including transcriptomic reprogramming and metabolic adjustments. Different types and organellar origins of ROS can trigger specific nuclear transcriptomic responses as shown by genetic analysis of individual signaling pathways (De Clercq *et al.*, 2013; Dogra *et al.*, 2017) as well as meta-analysis of transcriptome data from wild type and mutant plants exposed to exogenous ROS or ROS-inducing treatments (Willems *et al.*, 2016). For example, PSII-derived $^1O_2$ induces a distinct subset of nuclear genes involved in the regulation of cell death, jasmonic acid signaling and PSII repair collectively termed Singlet Oxygen Responsive Genes [SORGs; (Chan *et al.*, 2016b)]; with two different $^1O_2$–responsive signaling pathways responsible for regulation of different SORG subsets depending on the severity of the imposed stress [further discussed below; (Kim *et al.*, 2012; Ramel *et al.*, 2012; Dogra *et al.*, 2017)]. Another class of nuclear genes termed Plastid Redox Associated Nuclear Genes (PRANGs) comprise of various stress-associated genes including antioxidant enzymes and respond primarily to PSI H_2O_2-associated signaling (Chan *et al.*, 2016b). Similarly, mitochondrial ROS-inducing treatments induce a distinct class of genes termed Mitochondrial Dysfunction Stimulon (MDS) genes whose functions include mitochondrial ROS homeostasis (De Clercq *et al.*, 2013). Yet, organellar ROS responses are not limited to the organelle in which ROS are generated: as further discussed below, chloroplast ROS-associated signals can

have far-ranging impacts including on cytosolic ROS detoxification, endoplasmic reticulum protein homeostasis, and cell death.

With most of the ROS production occurring in the organelles and barring the relatively longer-lived H_2O_2, their short half-lives preclude ROS from being direct stress signals that travel (long-distance) from their production sites to elicit stress-responsive gene expression induction in the nucleus. Although H_2O_2 can be transferred directly from the chloroplasts to the nucleus (Exposito-Rodriguez *et al.*, 2017), this mechanism on its own does not confer sufficient specificity, because the organellar origin of the nuclear H_2O_2 cannot be distinguished. Yet, the nuclear transcriptomic responses to peroxisomal and chloroplastic H_2O_2 are clearly distinct (Sewelam *et al.*, 2014), indicating organelle-specific ROS-induced signaling pathways and/or ROS-independent mechanisms. In the past decades, several signaling pathways emanating from organelles have been shown to be crucial for the adjustment and/or repair of the organelles during environmental stress. These pathways are termed organellar "operational control" retrograde signaling [first described by Pogson *et al.* (2008); reviewed in Dietz *et al.* (2016); Leister (2017)]. These signaling pathways share the common trait of being responsive to, or associated with, ROS.

Chloroplastic ROS-induced retrograde signaling

As described above, $^1O_2$ is commonly produced at PSII and quenched by pigments, including β-carotene bound to the reaction centers of photosystems embedded in the thylakoid membrane. β-carotene itself is oxidized by $^1O_2$, giving rise to various cleavage products including β-cyclocitral (β-CC) and dihydroactinidiolide (Ramel *et al.*, 2012), which act as chloroplast retrograde signals during excess light stress (Fig. 2). Upon production, β-CC can induce $^1O_2$-specific stress responses, such as the up-regulation of the glutathione transferase class tau 5 (GSTU5) and mitogen-activated protein kinase 18 (MAPKKK18) (Ramel *et al.*, 2012). The β-CC signaling cascade proceeds at least partially via the RNA-binding protein methylene blue sensitivity 1 (MBS1) (Shumbe *et al.*, 2017) and intersects with various stress pathways, such as the xenobiotic detoxification response (D'Alessandro *et al.*, 2018) and the salicylic acid (SA) signaling (Lv *et al.*, 2015) for photooxidative stress acclimation. Interestingly, β-CC rapidly dissolves into β-cyclocitric acid (β-CCA), which only partially overlaps with the former in terms of regulated genes (Fig. 2), suggesting distinct functions for these two closely related metabolites (D'Alessandro *et al.*, 2019). Although multiple

494 downstream effectors of β-CC have been characterized, direct sensor(s)/receptor(s) of β-CC
495 are yet to be identified.

496 Recently another $^1O_2$-specific stress sensor located on the thylakoid membrane of
497 chloroplasts has been characterized, namely the EXECUTER 1 (EX1) protein (Wang *et al.*,
498 2016; Dogra *et al.*, 2019). EX1 is associated with PSII in the grana margin (Fig. 2) and can
499 undergo a posttranslational modification (PTM) by $^1O_2$ at the Trp643 residue of its singlet
500 oxygen sensor (SOS) domain. This PTM step is critical for initiating EX1 degradation by the
501 thylakoid-membrane bound FtsH2 metalloprotease, in which the protease domain faces the
502 stromal side of thylakoids (Kato and Sakamoto, 2009) for activation of downstream $^1O_2$
503 signaling (Dogra *et al.*, 2017; Dogra *et al.*, 2019). It is possible that the as yet-unidentified
504 degradation product(s) of EX1 act(s) as a retrograde signal that triggers $^1O_2$-responsive gene
505 activation in the nucleus, although experimental validation is still required. Although signals
506 derived both from β-CC and EX1-FtsH2 are triggered by $^1O_2$, they originate from the grana
507 core and margin, respectively. This spatial difference of $^1O_2$ generation may confer specificity
508 in the cellular response, because β-CC and EX1 activate different sets of $^1O_2$-responsive genes
509 in the nucleus with only a minimal overlap between the gene sets (Dogra *et al.*, 2017). An
510 alternative explanation is provided by the environmental context, since β-CC is associated
511 with cell-death promoting under high light stress conditions while EX1-mediated signaling
512 can occur at lower light intensities (Kim *et al.*, 2012).

513 Methylerythritol cyclodiphosphate (MEcPP – also abbreviated as MEcDP in some
514 literature) is another chloroplastic stress retrograde signal that accumulates during wounding
515 and high-light stresses (Xiao *et al.*, 2012). MEcPP is an intermediate metabolite of the
516 methylerythritol phosphate (MEP) pathway for isoprenoid biosynthesis in plastids (Fig. 2).
517 MEcPP accumulation is probably responsive to ROS, because the MEcPP-degrading enzyme
518 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) contains a redox-sensitive iron-
519 sulfur cluster at its active site (Seemann *et al.*, 2005) and bacterial HDS is known to be redox
520 regulated (Ostrovsky *et al.*, 1998). Extensive work on the *hds* mutant shows that MEcPP
521 activates various stress pathways via the nuclear transcription factor CAMTA3, including
522 plastidial radical scavenging and the endoplasmic reticulum (ER)-unfolded protein response
523 (Benn *et al.*, 2016). Over-accumulated MEcPP also impacts on growth-regulating pathways
524 (de Souza *et al.*, 2017; Wang *et al.*, 2017; Jiang *et al.*, 2018; Jiang *et al.*, 2020). Intriguingly,
525 quantification of MEcPP accumulation in isolated chloroplasts versus whole-leaf extracts of
526 oxidatively stressed spinach (*Spinacia oleracea*) revealed that the bulk of the MEcPP remains
527 in the chloroplasts (Rivasseau *et al.*, 2009). If this predominantly chloroplast-localized

accumulation of MEcPP were evolutionarily conserved across plants, it could suggest that either (i) low-abundance MEcPP in the cytosol is rapidly and sensitively sensed by high-affinity receptor(s), or (ii) MEcPP is actually perceived within chloroplasts and its information of chloroplast stress relayed to the nucleus by unknown component(s). Examining these hypothetical possibilities could provide novel insights into this pathway.

Drought and high light stresses both induce accumulation of the chloroplast retrograde signal 3'-phosphoadenosine-5'-phosphate (PAP) via oxidative inactivation of its catabolic enzyme SAL1 (Fig. 2) (Estavillo *et al.*, 2011; Chan *et al.*, 2016a). Interestingly, although PAP degradation by the redox-sensitive SAL1 may occur in both chloroplasts and mitochondria due to the dual localization of SAL1, PAP synthesis is thought to occur primarily in the cytosol and Golgi, based on the predicted localization of its biosynthetic enzymes, the sulfotransferases (SOTs). The SOTs transfer a sulfonyl group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to other acceptor molecules, such as desulfoglucosinolates, thereby releasing PAP as a by-product (Chan *et al.*, 2019). The high polarity of PAP prevents it from crossing the organelle outer membrane by diffusion. Rather, PAP traverses the organellar membrane via two PAP transporters in *Arabidopsis*, namely the PAPS/PAP transporter 1 (PAPST1) that is localized to the plastid envelope membrane and thylakoids (Gigolashvili *et al.*, 2012) and the PAPST2 in the plastidial and/or mitochondrial membranes in a tissue-specific manner (Ashykhmina *et al.*, 2019). Both transporters are antiporters that can exchange any two of either PAPS, PAP, ATP or ADP across the organelle membrane. PAPSTs probably play crucial roles in regulating the PAP-mediated signaling, because the PAP biosynthesis sites are uncoupled from those of its degradation. How these transporters fit into the PAP signaling framework is not fully understood (Chan *et al.*, 2019). The PAP movement from the cytosol into the nucleus is thought to proceed via passive diffusion (Estavillo *et al.*, 2011). In the nucleus, PAP inhibits the activity of 5'-3' exoribonucleases (XRNs), which leads to altered RNA Polymerase II activity, thereby inducing the up-regulation of many stress-responsive genes, including chloroplast- and cytosol-targeted APXs (Estavillo *et al.*, 2011; Crisp *et al.*, 2018). Interestingly, PAP also affects ROS metabolism at the apoplast of guard cells, most likely by influencing the activity of NADPH oxidases (Pornsiriwong *et al.*, 2017).

Are there other ROS-responsive/dependent chloroplast signals, whether protein or metabolite-based? There is increasing evidence that some chloroplast-localized proteins can be directly translocated from the chloroplasts to the nucleus (Krupinska *et al.*, 2020). In response to the tobacco mosaic virus effector protein p50, ROS accumulates in chloroplasts.

Concurrently, the chloroplastic defense protein N receptor interacting protein 1 (NRIP1) recognizes p50 and is translocated with p50 to the nucleus, possibly via stromules (Caplan *et al.*, 2015). In another example, plants expressing a tagged version of the DNA-binding protein WHIRLY1 specifically in the chloroplast genome, thus ruling out dual targeting of cytosolically translated WHIRLY1, also show plastidial WHIRLY1 translocation to the nucleus and coincided with the increased expression of pathogenesis-related genes, consistent with their promoters as targets for WHIRLY1 binding. WHIRLY1 translocation has been proposed to be regulated by changes in the chloroplast redox state, for example during pathogen infection (Foyer *et al.*, 2014) when ROS also overaccumulates (Caplan *et al.*, 2015). It will be important to fully elucidate the mechanism(s) by which ROS regulate the translocation of these proteins, given their crucial roles.

Similarly to how ROS can react with β-carotene to produce electrophilic β-CC, ROS can also react with phospholipids -key components of cellular and organellar membranes- to form lipid peroxides and/or oxylipins (Farmer and Mueller, 2013; Mano *et al.*, 2019). Lipid peroxides contribute to a variety of reactive electrophile species (RES) or reactive carbonyl species (RCS) that can further react with proteins and covalently modify them (Winger *et al.*, 2005; Winger *et al.*, 2007). Lipid peroxides and their derivatives can trigger various physiological responses in plants and algae, including alteration in redox homeostasis, activation of programmed cell death, and changes in gene expression in the nucleus (Biswas and Mano, 2015; Roach *et al.*, 2018). Interestingly, treatment of plants and/or algae with moderate level of oxylipins/RES/RCS improved their tolerance toward stresses (e.g., light and heat) (Roach *et al.*, 2018; Monte *et al.*, 2020). Consequently, there is an increasing appraisal for oxylipins/RES/RCS to be considered also as players in ROS/organelle retrograde signaling; although conclusive evidence is still required (Farmer and Mueller, 2013; Alché, 2019; Mano *et al.*, 2019; Muñoz and Munné-Bosch, 2020).

Mitochondrial ROS-induced signaling

In contrast to chloroplast ROS-induced signaling pathways, which are rather well characterized to a certain extent, detailed knowledge on equivalent mitochondrial pathways remain sparse. Only one major pathway has been elucidated to date, involving the *Arabidopsis* NAC domain-containing protein 13 (ANAC013) (De Clercq *et al.*, 2013), ANAC017 (Ng *et al.*, 2013; Meng *et al.*, 2019), and radical-induced cell death 1 (RCD1)

(Shapiguzov *et al.*, 2019). Perturbations of the mETC leads to translocation of the ER-tethered ANAC013 and ANAC017 transcription factors to the nucleus via the action of unidentified proteases (Fig. 2). In the nucleus, ANAC013 and ANAC017 regulate the expression of mitochondrial dysfunction stimulon (MDS) genes, which encode, among others, *AOX1a* that is crucial for mitochondrial ROS detoxification [reviewed in Waszczak *et al.* (2018); Dourmap *et al.* (2020); Mielecki *et al.* (2020); Wang *et al.* (2020b)]. RCD1 is a redox-sensitive WWE domain-containing nuclear protein. Under unstressed conditions, monomeric RCD1 physically interacts with and sequesters ANAC013/ANAC017. Under stress, ROS-induced oligomerization of RCD1 releases this inhibition, allowing ANAC013/ANAC017 to regulate the *MDS* gene expression (Shapiguzov *et al.*, 2019).

Interestingly, RCD1-ANAC013/017 were proposed to intersect with SAL1-PAP-mediated chloroplast retrograde signaling based on similarities in MDS gene expression and oxidative stress phenotypes of the respective mutants (Van Aken and Pogson, 2017; Shapiguzov *et al.*, 2019). Although the exact molecular mechanism behind this interaction remains a mystery, these findings suggest that chloroplast and mitochondrial retrograde signaling converge in the nucleus (Wang *et al.*, 2020b). One possible purpose of such convergence may be the coordination of transcriptional changes to fine-tune the overall cellular energy metabolism by readjusting the capacity of both organelles according to environmental cues.

Peroxisomal ROS-induced signaling

The existence of peroxisome-derived retrograde signaling has been proposed previously (Foyer and Noctor, 2003). This hypothesis is plausible because peroxisomes are the sources of various important signaling molecules, including β-oxidation--derived molecules, such as some hormone (jasmonic acid, auxin, and SA) derivatives, ROS, reactive nitrogen species (RNS), and components of redox buffers that convey the change in the peroxisomal redox state (Baker *et al.*, 2006; Nyathi and Baker, 2006). Peroxisomal ROS triggers specific transcriptional changes in the nucleus (Sewelam *et al.*, 2014). Reported changes in the peroxisomes under oxidative stress include altered morphology (shape and size), mobility, and numbers (biogenesis vs pexophagy/turnover) (Wang *et al.*, 2015; Jaipargas *et al.*, 2016; Rodríguez-Serrano *et al.*, 2016; Su *et al.*, 2019; Pan *et al.*, 2020; Sandalio *et al.*, 2021). These features hint at the possibility of peroxisomal ROS signaling, leading to changes in the nuclear gene expression that readjust peroxisome properties for acclimation, corresponding

with the definition of retrograde signaling (Chan *et al.*, 2016b). However, no ROS-induced peroxisome-to-nucleus retrograde signaling pathway has been described to date (Fig. 2).

Whereas knowledge of peroxisomal ROS signaling is still fragmentary, in the past decade loss-of-function studies with the peroxisomal *catalase 2* (*cat2)* mutant have revealed candidate pathways and components. Conditional overproduction of peroxisomal ROS and enhanced cell death in *cat2* are associated with altered glutathione redox state and activation of the hypersensitive response via SA (Queval *et al.*, 2007; Chaouch *et al.*, 2010). Forward and reverse genetic screens for suppressors of the *cat2* photorespiratory ROS-related phenotypes also identified crucial roles for hormonal signaling and various transcription factors (Kaurilind *et al.*, 2015; Kerchev *et al.*, 2016; Waszczak *et al.*, 2016). Interestingly the different processes mentioned above, albeit merely correlative, have also been linked with different chloroplast retrograde signaling pathways; for example, SA with β-CC signaling, auxin with both MEcPP and PAP, and glutathione redox state with PAP (Lv *et al.*, 2015; Jiang *et al.*, 2018; Phua *et al.*, 2018). The biggest challenge remains to identify the actual peroxisomal retrograde signal(s), if they exist, and elucidate the pathways in which they function. As part of this process, a starting point could be to examine the extent to which peroxisomal ROS diffuses into the cytosol and nuclei to affect redox couples and/or signaling proteins directly.

Perspectives

In the past decade, although our understanding of ROS metabolism and signaling in different plant organelles has progressed, several questions remain. First, do the different retrograde signals function in concert with, independently of, or even antagonistically to each other? Second, how do these signals intersect with the nuclear H_2O_2 perception directly transferred from chloroplasts? To address this question, the direct impact of the chloroplast-to-nucleus H_2O_2 transfer on the nuclear transcriptome needs to be determined as well. Third, which are the genes encoding the missing components in the organellar ROS signaling, including potential ROS/redox sensors in mitochondria and peroxisomes? In the specific case of ANAC013/017-mediated mitochondrial signaling, an important related question is how is mitochondrial ROS perception transduced to the ER, where the ANAC proteins reside. Fourth, are the different organelle ROS signaling networks interconnected in the same manner

as the metabolic link between chloroplasts, mitochondria, and peroxisomes; and if so, which are the missing components?

Each of these questions could be partially addressed by identifying signaling proteins responsive to ROS from the different organelles. Recent technological advancements enabled *in vivo* trapping and identification of oxidized cysteines in different subcellular compartments upon oxidative stresses in cultured *Arabidopsis* cells (De Smet *et al.*, 2019; Huang *et al.*, 2019; Wei *et al.*, 2020). These approaches revealed potentially crucial roles for ROS-mediated cysteine oxidation in RNA processing and amino acid metabolism (De Smet *et al.*, 2019; Huang *et al.*, 2019) and provide a complementary strategy to the conventional biochemical characterization of signaling proteins identified from genetic screens (Lee *et al.*, 2007; Estavillo *et al.*, 2011; Chan *et al.*, 2016a; Dogra *et al.*, 2019). Nevertheless, a major technical challenge lies in the improvement of the sensitivity of such cysteine-trapping approaches to capture novel organellar retrograde redox sensors/signals. Transfer of the trapping technology from cultured cells to intact plants will also enhance the physiological relevance of any identified ROS sensor(s).

Another possible avenue for future breakthroughs in ROS signaling is to break down the complexity of the studied biological system. A plant leaf is composed of several highly specialized cell types that can have diverse ROS signaling pathways, as evidenced by the contribution of apoplastic ROS production specifically by vascular cells, but not other cell types, to systemic stress signaling (Zandalinas *et al.*, 2020). It is noteworthy that transcriptomics of isolated single cells from a complex organ is now feasible for *Arabidopsis* (Wendrich *et al.*, 2020). Complementary approaches such as using cultured *Arabidopsis* cells derived from a single tissue type (Van Leene *et al.*, 2007) and/or shifting completely to a unicellular model organism that houses both chloroplasts and mitochondria, such as *Chlamydomonas reinhardtii*, could help identify missing ROS and organellar retrograde signaling pathways that are "diluted" by whole-leaf analyses. For instance, Shao *et al.* (2013) successfully utilized *C. reinhardtii* to detect new players in ROS signaling in *Arabidopsis*, but in other cases the identified proteins had been lost in higher plants (Fischer *et al.*, 2012; Wakao *et al.*, 2014). As homologous proteins for the *Arabidopsis* organellar signaling proteins SAL1, HDS1, and EX1 are conserved in *C. reinhardtii*, it may be interesting to assess the extent to which the principles and components of ROS signaling, studied extensively in *Arabidopsis*, are actually maintained across the green lineage. In that respect, mutants affected in NPQ represent interesting tools to understand the response to light stress and signaling from the chloroplast. The photosynthetic response of mutants affected in qT

(*lhcsr* mutants), qE (*stt7* mutants), or both pathways to various light conditions (high light or fluctuating light) has been characterized in detail [e.g. Peers *et al.* (2009); Allorent *et al.* (2013); Girolomoni *et al.* (2019)]. Although the retrograde signaling leading to changes in the *LHCSR3* expression has not been elucidated yet, the involvement of chloroplastic Ca^{2+} variations and redox imbalance has been proposed [reviewed in Rea *et al.* (2018)]. In addition, the availability of respiratory deficient mutants (Salinas *et al.*, 2014), and the existence of double mutants affected in both respiration and photosynthetic activity (Cardol *et al.*, 2009; Massoz *et al.*, 2017; Larosa *et al.*, 2018) could help decipher retrograde signaling from mitochondria and its connection with the chloroplast.

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Conflict of interest

The authors declare no conflict of interest.

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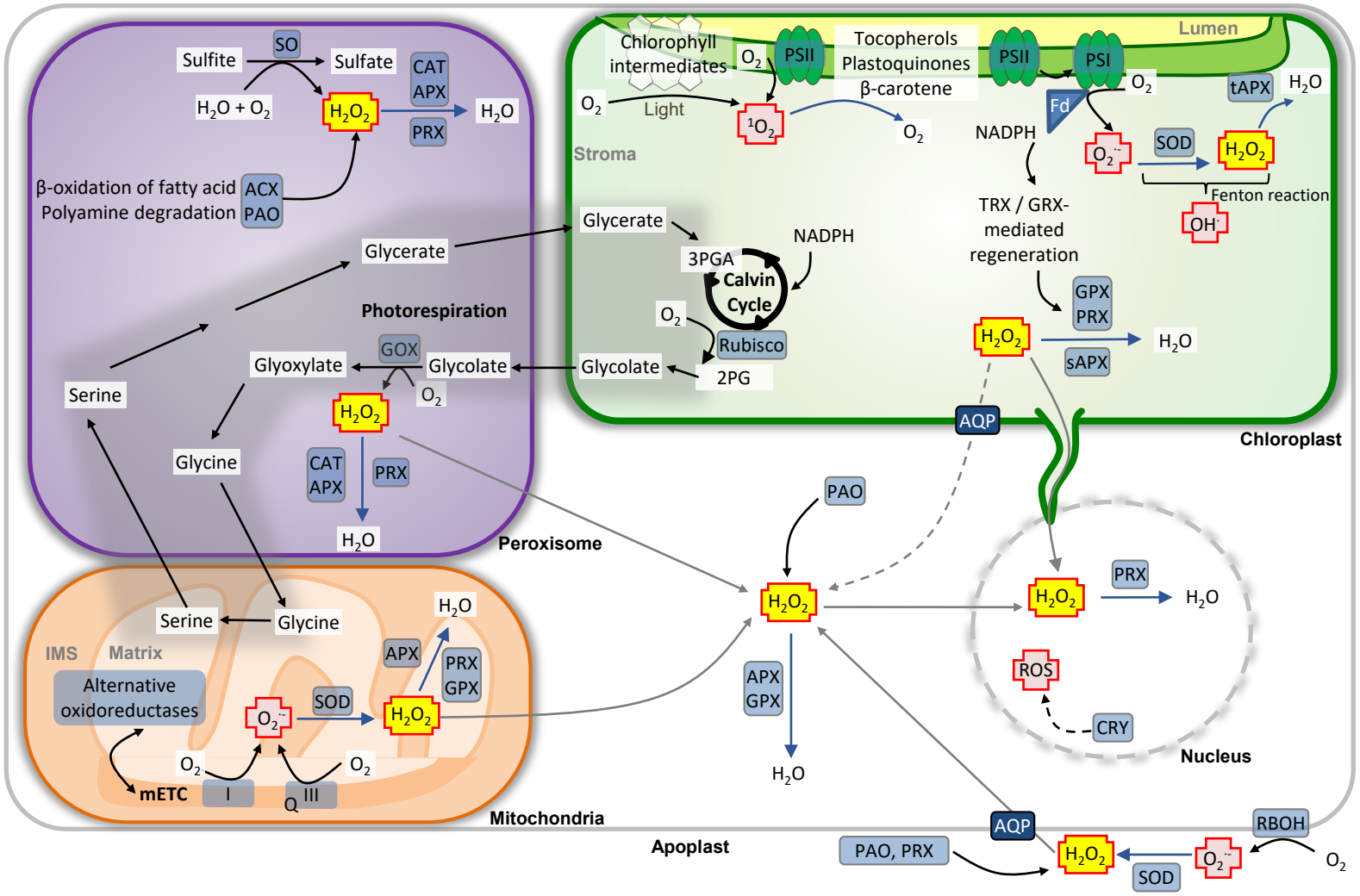
Figure Legends

Fig. 1. Summary of the major ROS-producing and –quenching pathways in plant cells. In chloroplasts, the photosynthetic electron transport chain can produce reactive oxygen species (ROS) at both Photosystem II (PSII) and Photosystem I (PSI). At PSII, excited chlorophyll molecules transfer energy to triplet oxygen to produce singlet oxygen ($^1O_2$), which can be quenched by multiple pigment molecules. $^1O_2$ can also be produced when the photosensitizing biosynthetic intermediates of chlorophyll are exposed to light. At PSI, electron leakage to O_2 results in superoxide ($O_2^{\cdot-}$) formation, which is converted to hydrogen peroxide (H_2O_2) either via the action of SODs or by spontaneous dismutation. H_2O_2 can attack the iron (Fe) co-factor of SODs to form hydroxyl radicals ($OH^{\cdot}$) – also known as Fenton reaction. The incorporation of O_2 rather than CO_2 by Rubisco in chloroplasts leads to induction of the photorespiratory pathway; glycolate from chloroplasts is exported into peroxisomes where its conversion into glyoxylate is a major source of H_2O_2 in this compartment. Other enzymatic processes, such as sulfite and fatty acid oxidation, as well as polyamine degradation, also contribute to peroxisomal H_2O_2 production, Polyamine degradation by polyamine oxidases (PAO) also produces H_2O_2 in the cytosol. In mitochondria, Complexes I and III of the mitochondrial electron transport chain (mETC) both produce $O_2^{\cdot-}$ which is then converted to H_2O_2. The apoplast is another major source of ROS; here the respiratory burst oxidase homologs (RBOH) proteins catalyse $O_2^{\cdot-}$ production. Similar to the other compartments, SODs are thought to be responsible for conversion of $O_2^{\cdot-}$ to H_2O_2 in the apoplast, with PAO and peroxiredoxins (PRX) also contributing to apoplastic H_2O_2 production. Cryptochromes (CRY) have been implicated in ROS production in the nucleus, although the exact type of ROS being produced is unclear. H_2O_2 movement between subcellular compartments can be facilitated by aquaporins (AQP). The detoxification of H_2O_2 to water is achieved via similar mechanisms in the different subcellular compartments, with ascorbate peroxidase (APX), glutathione peroxidase (GPX), and/or PRX being the main enzymes involved. GPX and PRX utilise redox-sensitive cysteines in their active site to convert H_2O_2 to water; the oxidized cysteines are regenerated by thioredoxins and glutaredoxins which are themselves regenerated by other reductases using reducing power from PSI. In the peroxisomes, catalase (CAT) is an additional, and indeed the dominant, H_2O_2-detoxification enzyme. In the mitochondria, multiple alternative oxidoreductases, such as Alternative Oxidase 1a (AOX1a) also contribute to decreasing $O_2^{\cdot-}$ and H_2O_2 production by removing excess electrons from the mETC. All black curved arrows denote electron transfer, straight black arrows indicate enzymatic

reactions, grey arrows show movement, blue arrows indicate ROS detoxification steps and dashed lines denote putative events with components/players yet to be experimentally demonstrated. Other abbreviations: acyl-CoA oxidases (ACX), ferredoxin (Fd), glycolate oxidase (GOX), intermembrane space (IMS) stromal ascorbate peroxidase (sAPX), sulfite oxidase (SO), thylakoidal ascorbate peroxidase (tAPX), ubiquinone/ubiquinol (Q), 3-phosphoglycerate (3-PGA), 2-phosphoglycolate (2-PG). Organelles are not drawn to scale.

Fig. 2. ROS-induced organelle-to-nucleus retrograde signalling pathways. In the chloroplast, both Photosystem II (PSII) and PSI-induced ROS are capable of activating different chloroplast-to-nucleus retrograde signals. Singlet oxygen ($^1O_2$) production at the grana margins leads to oxidation of the Executer 1 (Ex1) protein, which is then cleaved by the thylakoidal FtSH2 protease. An unknown Ex1 peptide fragment then initiates signalling to the nucleus to activate expression of a subset of Singlet Oxygen Responsive Genes (SORGs I) associated with regulation of cell death. Conversely, $^1O_2$ production by the PSII reaction center at the grana core is sensed via oxidative cleavage of β-carotene to produce β-cyclocitral (β-CC) which is a volatile retrograde signal. β-CC can be rapidly converted to β-cyclocitric acid (β-CCA). Both β-CC and β-CCA regulate the expression of a second subset of SORGs (SORGs II), which is distinct to those regulated by EX1. The regulation of SORGs II by β-CC is dependent on the cytosolic protein Methylene Blue Sensitive 1 (MBS1), whereas β-CCA functions independently of MBS1. β-CC also activates the expression of genes involved in xenobiotic detoxification responses. ROS produced from PSI are thought to decrease the activities of 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) and SAL1 via redox regulation, thereby enabling accumulation of their respective substrates 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) and 3'-phosphoadenosine 5'-phosphate (PAP). Both of these metabolites function as retrograde signals which target a broad class of genes independent of $^1O_2$ but otherwise responsive to oxidative stress in chloroplasts termed Plastid Redox Associated Nuclear Genes (PRANGs). MEcPP –mediated signalling proceeds in the nucleus at least in part via the CAMTA transcription factor, but the mechanisms for intracellular transport and nuclear perception of MEcPP are still unknown. PAP transport between the chloroplast and the cytosol is mediated by PAPS/PAP transporter 1 (PAPST1) and PAPST2 transporters. In the nucleus, PAP binds to exoribonuclease enzymes (XRNs), which alters RNA Polymerase II (Pol II) activity thereby enabling up-regulation of a large number of PRANGs. Direct transfer of H_2O_2 from chloroplasts to the nucleus has been observed and is proposed to be a signalling mechanism, but nuclear receptors for H_2O_2 are

currently unknown. ROS overproduction in mitochondria leads to activation of an unknown protease which releases the ANAC017 / ANAC013 transcription factors from their ER tethering. The truncated ANAC017 / ANAC013 proteins translocate to the nucleus, where they activate expression of Mitochondrial Dysfunction Stimulon (MDS) genes including Alternative Oxidase 1a (AOX1a). This mitochondrial signalling pathway is normally suppressed in the nucleus by RCD1, but activated during mitochondrial stress due to redox-mediated inactivation of RCD1 (possibly by nuclear H_2O_2). Another proposed regulator for this mitochondrial pathway is PAP, although the mechanism is still unclear. Mitochondria also contain PAP and SAL1, but it is not clear if mitochondrial SAL1 is similarly redox-regulated to enable PAP to function as a mitochondrial retrograde signal. Peroxisomes are sources of various potential signalling molecules, but their connection to peroxisomal ROS and hypothetical retrograde signalling from peroxisomes is still not clear. Red arrows/lines denote ROS-induced signalling events, blue arrow indicates ROS detoxification step, dashed lines denote putative events with components/players yet to be experimentally demonstrated, red fonts denote ROS-sensors, yellow boxes highlight signalling molecules. Other abbreviations: adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), 4-hydroxyl-3-methyl-butenyl 1-diphosphate (HMBPP), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), reactive nitrogen species (RNS). Organelles are not drawn to scale.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Peroxisome
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O_2
SOD

Fig. 1. Summary of the major ROS-producing and –quenching pathways in plant cells. In chloroplasts, the photosynthetic electron transport chain (ETC) can produce reactive oxygen species (ROS) at both photosystem II (PSII) and photosystem I (PSI). At PSII, excited chlorophyll molecules transfer energy to triplet oxygen to produce singlet oxygen ($^1O_2$), which can be quenched by multiple pigment molecules. $^1O_2$ can also be produced when the photosensitizing biosynthetic intermediates of chlorophyll are exposed to light. At PSI, electron leakage to O_2 results in superoxide ($O_2^{\cdot-}$) formation, which is converted to hydrogen peroxide (H_2O_2) either via the action of superoxide dismutases (SODs) or by spontaneous dismutation. H_2O_2 can attack the iron (Fe) cofactor of SODs to form hydroxyl radicals ($OH^{\cdot}$) – also known as Fenton reaction. The incorporation of O_2 rather than CO_2 by Rubisco in chloroplasts induces the photorespiratory pathway; glycolate from chloroplasts is exported into peroxisomes, where it is converted into glyoxylate and so is a major source of H_2O_2 in this compartment. Other enzymatic processes, such as sulfite and fatty acid oxidation, as well as polyamine degradation, also contribute to peroxisomal H_2O_2 production, Polyamine degradation by polyamine oxidases (PAOs) also produces H_2O_2 in the cytosol. In mitochondria, complexes I and III of the mitochondrial (m)ETC both produce $O_2^{\cdot-}$ that is then converted to H_2O_2. The apoplast is another major source of ROS; here the respiratory burst oxidase homolog (RBOH) proteins catalyze $O_2^{\cdot-}$ production. Similarly to the other compartments, SODs are thought to be responsible for the conversion of $O_2^{\cdot-}$ to H_2O_2 in the apoplast, with PAOs and peroxiredoxins (PRXs) also generating apoplastic H_2O_2. Cryptochromes (CRY) have been implicated in ROS production in the nucleus, although the exact ROS type is unclear. H_2O_2 movement between subcellular compartments can be facilitated by aquaporins (AQPs). H_2O_2 is detoxified to water via similar mechanisms in the different subcellular compartments, with ascorbate peroxidase (APX), glutathione peroxidase (GPX), and/or PRX as the main enzymes involved. GPX and PRX utilize redox-sensitive cysteines in their active site to convert H_2O_2 to water; the oxidized cysteines are regenerated by thioredoxins and glutaredoxins, which are themselves regenerated by other reductases by means of the PSI reducing power. In the peroxisomes, catalase (CAT) is an additional, and indeed the dominant, H_2O_2 detoxification enzyme. In the mitochondria, multiple alternative oxidoreductases, such as Alternative Oxidase 1a (AOX1a), also decrease $O_2^{\cdot-}$ and H_2O_2 production by removing excess electrons from the mETC. All black curved arrows denote electron transfer, straight black arrows indicate enzymatic reactions, grey arrows show movement, blue arrows indicate ROS detoxification steps and dashed lines denote putative events with components/players yet to be experimentally demonstrated. Abbreviations: ACX, acyl-CoA oxidases; Fd, ferredoxin, GOX, glycolate oxidase; IMS, intermembrane space; sAPX, stromal ascorbate peroxidase; SO, sulfite oxidase; tAPX, thylakoidal ascorbate peroxidase; Q, ubiquinone/ubiquinol; 3PGA, 3-phosphoglycerate; 2PG, 2-phosphoglycolate. Organelles are not drawn to scale.

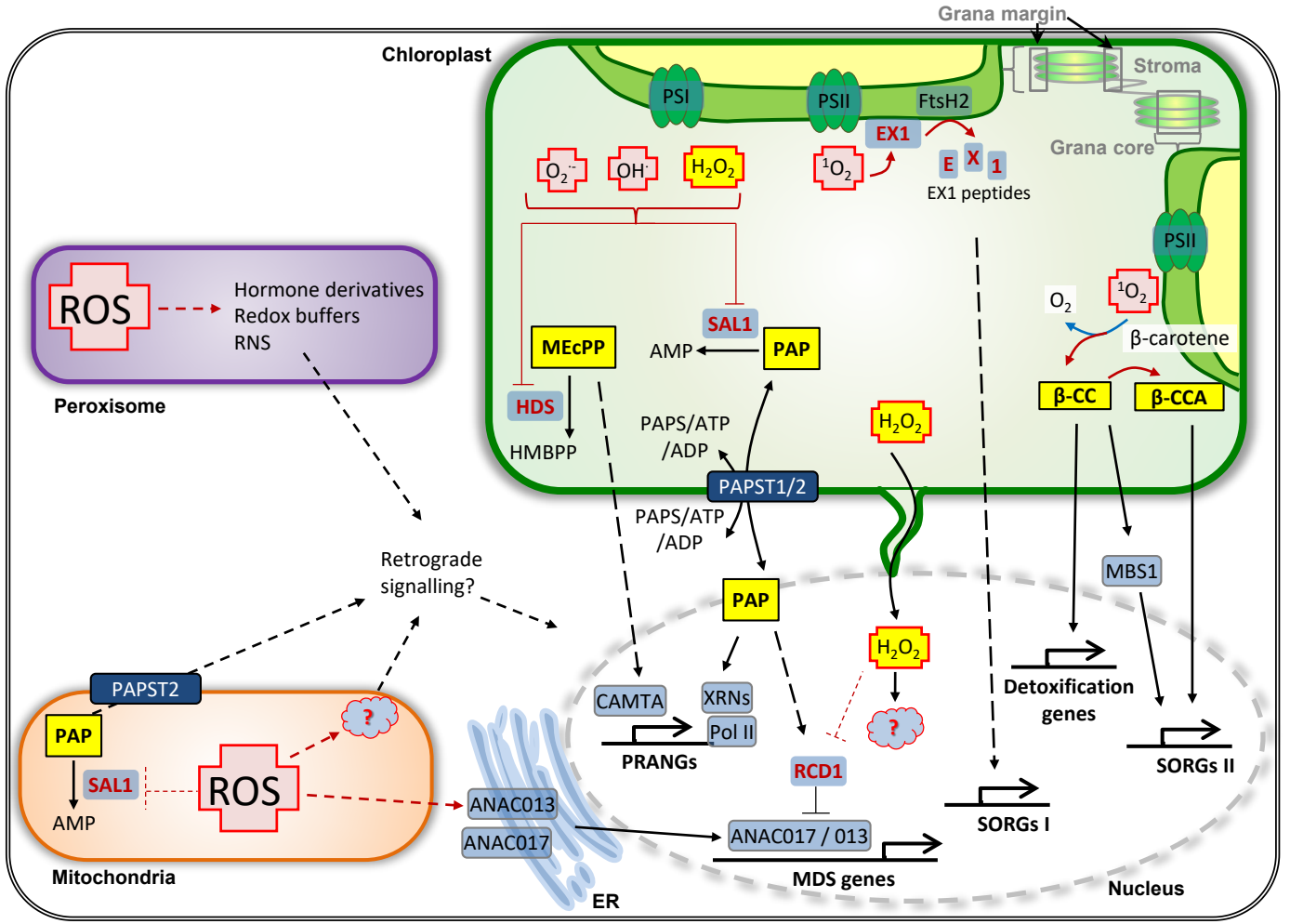
Grana margin
Chloroplast
Stroma
PSI
PSII
FtsH2
EX1
Grana core
$O_2^{\cdot-}$
$OH^{\cdot}$
H_2O_2
$^1O_2$
E X 1
EX1 peptides
PSII
O_2
$^1O_2$
β-carotene
β-CC
β-CCA
ROS
Hormone derivatives
Redox buffers
RNS
Peroxisome
SAL1
MEcPP
AMP
PAP
HDS
HMBPP
PAPS/ATP
/ADP
H_2O_2
PAPST1/2
PAPS/ATP
/ADP
Retrograde
signalling?
PAP
MBS1
H_2O_2
PAPST2
PAP
CAMTA
XRNs
Pol II
PRANGs
Detoxification
genes
SAL1
ROS
AMP
Mitochondria
ANAC013
ANAC017
RCD1
SORGs II
ANAC017 / 013
SORGs I
MDS genes
ER
Nucleus

Fig. 2. ROS-induced organelle-to-nucleus retrograde signaling pathways. In the chloroplast, both photosystem II (PSII) and PSI-induced ROS can activate different chloroplast-to-nucleus retrograde signals. Singlet oxygen ($^1O_2$) production at the grana margins leads to oxidation of the Executer 1 (EX1) protein, which is cleaved by the thylakoidal FtSH2 protease. An unknown Ex1 peptide fragment initiates signaling to the nucleus to activate expression of a subset of Singlet Oxygen Responsive Genes (SORGs I) associated with the regulation of cell death. Conversely, $^1O_2$ production by the PSII reaction center at the grana core is sensed via oxidative cleavage of β-carotene to produce β-cyclocitral (β-CC), which is a volatile retrograde signal. β-CC can be rapidly converted to β-cyclocitric acid (β-CCA). Both β-CC and β-CCA regulate the expression of a second subset of SORGs (SORGs II), which is distinct to those regulated by EX1. The regulation of SORGs II by β-CC depends on the cytosolic protein methylene blue sensitive 1 (MBS1), whereas β-CCA functions independently of MBS1. β-CC also activates the expression of genes involved in xenobiotic detoxification responses. PSI-produced ROS are thought to decrease the activities of 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) and SAL1 via redox regulation, thereby enabling the accumulation of their respective substrates 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) and 3'-phosphoadenosine 5'-phosphate (PAP). Both these metabolites function as retrograde signals that target a broad class of genes independently of $^1O_2$, but are otherwise responsive to oxidative stress in chloroplasts, designated Plastid Redox Associated Nuclear Genes (PRANGs). MEcPP-mediated signaling proceeds in the nucleus at least in part via the CAMTA transcription factor, but the mechanisms for intracellular transport and nuclear perception of MEcPP are still unknown. PAP transport between the chloroplast and the cytosol is mediated by PAPS/PAP transporter 1 (PAPST1) and PAPST2. In the nucleus, PAP binds to exoribonuclease enzymes (XRNs) that alter the RNA Polymerase II (Pol II) activity, thereby up-regulating a large number of PRANGs. Although direct transfer of H_2O_2 from chloroplasts to the nucleus has been observed and has been proposed to be a signaling mechanism, the nuclear H_2O_2 receptors are currently unknown. ROS overproduction in mitochondria activates an unknown protease that releases the ANAC017/ANAC013 transcription factors from their endoplasmic reticulum (ER) tethering. The truncated ANAC017/ANAC013 proteins translocate to the nucleus, where they activate the expression of the Mitochondrial Dysfunction Stimulon (*MDS*) genes, including Alternative Oxidase 1a (*AOX1a*). This mitochondrial signaling pathway is normally suppressed in the nucleus by RCD1, but activated during mitochondrial stress due to redox-mediated inactivation of RCD1 (possibly by nuclear H_2O_2). Another proposed regulator for this mitochondrial pathway is PAP, although the mechanism is still unidentified. Mitochondria also contain PAP and SAL1, but it is unknown whether mitochondrial SAL1 is similarly redox regulated to enable PAP to function as a mitochondrial retrograde signal. Peroxisomes are sources of various potential signaling molecules, but their connection to peroxisomal ROS and hypothetical retrograde signaling from peroxisomes is still unclear. Red arrows/lines denote ROS-induced signaling events; the blue arrow indicates a ROS detoxification step; dashed lines denote putative events with components/players yet to be experimentally demonstrated; red fonts denote ROS sensors; yellow boxes highlight signaling molecules. Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; HMBPP, 4-hydroxyl-3-methyl-butenyl 1-diphosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; RNS, reactive nitrogen species. Organelles are not drawn to scale.