ROS-dependent signaling pathways in plants and algae exposed to high light: Comparisons with other eukaryotes.

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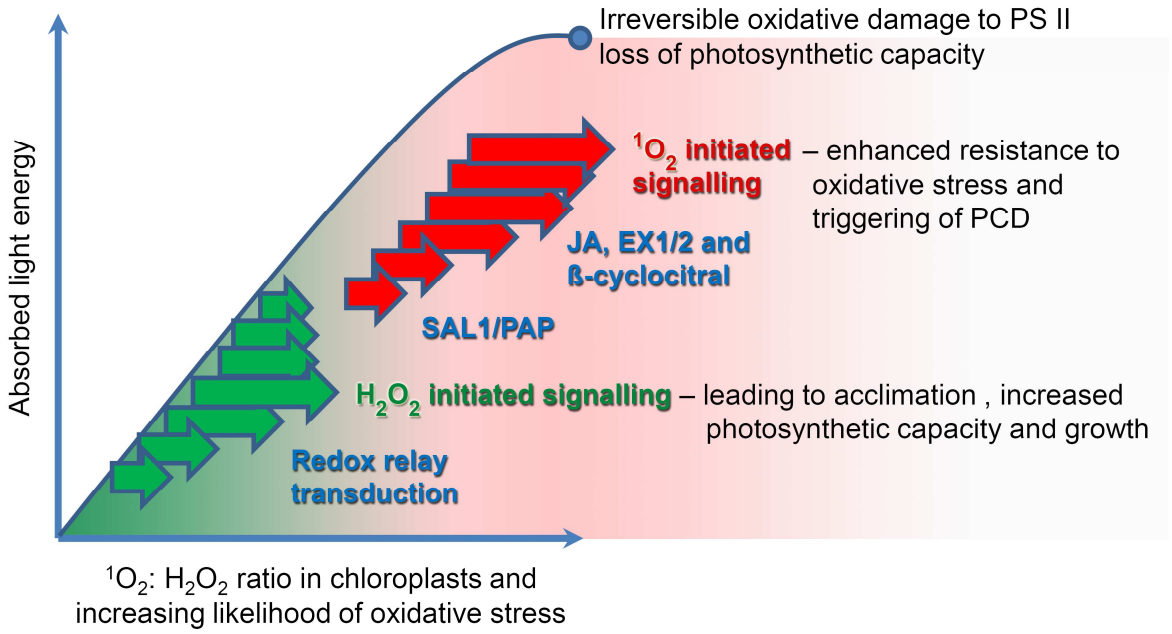
Abstract

Like all aerobic organisms, plants and algae co-opt reactive oxygen species (ROS) as signaling molecules to drive cellular responses to changes in their environment. In this respect, there is considerable commonality between all eukaryotes imposed by the constraints of ROS chemistry, similar metabolism in many subcellular compartments, the requirement for a high degree of signal specificity and the deployment of thiol peroxidases as transducers of oxidizing equivalents to regulatory proteins. Nevertheless, plants and algae carry out specialised signaling arising from oxygenic photosynthesis in chloroplasts and photoautotropism, which often induce an imbalance between absorption of light energy and the capacity to use it productively. A key means of responding to this imbalance is through communication of chloroplasts with the nucleus to adjust cellular metabolism. Two ROS, singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), initiate distinct signaling pathways when photosynthesis is perturbed. $^1O_2$, because of its potent reactivity means that it initiates but does not transduce signaling. In contrast, the lower reactivity of H_2O_2 means that it can also be a mobile messenger in a spatially-defined signaling pathway. How plants translate a H_2O_2 message to bring about changes in gene expression is unknown and therefore, we draw on information from other eukaryotes to propose a working hypothesis. The role of these ROS generated in other subcellular compartments of plant cells in response to HL is critically considered alongside other eukaryotes. Finally, the responses of animal cells to oxidative stress upon high irradiance exposure is considered for new comparisons between plant and animal cells.

Abstract figure



Abstract Figure: Summary of the main features of ROS-initiated chloroplast-to-nucleus retrograde signalling. PAP 3'-phosphoadenosine 5'-phosphate; JA jasmonic acid; EX1/2 EXECUTER 1 and EXECUTER 2.

Highlights

- An outline of the common features of deploying reactive oxygen species (ROS) as signalling molecules in all eukaryotes even when considering plant cells exposed to high light.
- The description of a possibly complete chloroplast-nucleus retrograde signalling route initiated by $^1O_2$.
- An up-to-date description of spatial components of chloroplast–to-nucleus retrograde communication, which solves many issues of signal specificity.
- Drawing on non-plant experimental systems, we outline hypotheses of how a H_2O_2 signal is propagated when it exits the chloroplast and arrives in the nucleus or the cytosol.
- A critical consideration of the role of ROS from other subcellular compartments to act in retrograde signalling in high light-exposed plant cells.

Keywords

Singlet oxygen, hydrogen peroxide, photosynthesis, chloroplast-to-nucleus communication, retrograde signalling, ROS-mediated signalling, high light, mitochondria, peroxisomes, microdomains, redox relay, thiol peroxidases, yeast, animal cells, plant cells

Abbreviations: ABA; abscisic acid; ABC; ATP binding cassette; ADP + Pi, adenosine diphosphate and inorganic phosphate; AOX, alternative oxidase; APX, ascorbate peroxidase; ATP, adenosine triphosphate; AQP, aquaporin; b6f, Cytochrome b6f complex; CysP, peroxidatic cysteine; CysP-OH, sulfenic acid form of CysP; CysR, resolving cysteine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EEE, excess excitation energy; ER; endoplasmic reticulum; FLV, flavodiiron; FRBM, Free Radical Biology & Medicine; GPX, glutathione peroxidase; H2O, water; H2O2, hydrogen peroxide; HL, high light; HOTE, 10-hydroxy- 8,12,15(E,Z,Z)octadecatrienoic acid; HSFA1d, Heat Shock Transcription Factor A1d; JA, jasmonic acid; LEF, linear electron flow; MDA, monodehydroascorbate radical; MDAR, monodehydroascorbate reductase; MEcPP, methyl erythritol cyclodiphosphate; mtETC, mitochondrial respiratory electron transport chain; NADPH, Nicotinamide adenine dinucleotide phosphate; OH., hydroxyl radical; O2, molecular (ground state) oxygen; 1O2, singlet oxygen; O2.-, superoxide anion; P680, primary oxidant of PSII; PAP, 3' phosphoadenosine 5' phosphate; PC, plastocyanin; PCD, programmed cell death; PET, photosynthetic electron transport; PQH2 plastoquinol; PRX, peroxiredoxin; PUFA, polyunsaturated fatty acid; PPFD, photosynthetically active photon flux density; PQ, photochemical quenching; PSI, photosystem I; PSII, photosystem II; PTOX, plastid terminal oxidase; roGFP2; redox-sensitive green fluorescent protein 2; ROS, reactive oxygen species; SOD, superoxide dismutase; WWC, water-water cycle.

Aims and limits of this Review

For plants, changes in light intensity, duration and quality govern every aspect of their life cycle and metabolism. In its very small way this review will focus on one important aspect; how leaves elicit signalling responses to increased light intensity, which presents both a threat of photo-damage to the photosynthetic apparatus, but also an opportunity to increase photosynthesis and therefore growth and fecundity, which we can term high light (HL) stress and acclimation respectively. The special issue of FRBM also sets the task of determining if there are implications for mammalian physiology in considering this most plant-specific of processes.

Therefore, while we focus this review on the chloroplast and its ability to transmit signals to the nucleus to bring about changes in gene expression in response to HL, we do draw on relevant mechanistic processes in other eukaryotic cells. We have done this, in part, by focussing on signalling associated primarily with the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which unless otherwise stated, we term ROS when considering them together.

There is a considerable body of research conducted on signalling from dysfunctional chloroplasts, which includes many of the first studies on chloroplast-to-nucleus retrograde signalling [1, 2]. One would expect that ROS would be highly implicated in signalling from disabled chloroplasts. This is the case in many of the *genomes uncoupled* mutants, which accumulate photodynamic intermediates of chlorophyll biosynthesis, which cause extensive oxidative stress in HL. Likewise, in wild type plants treated with the carotenoid biosynthesis inhibitor herbicide norflurazon or prokaryotic protein synthesis inhibitor lincomycin, disabled chloroplasts would likely produce ROS. However, it is difficult to extrapolate from such mutants and treatments to wild type plants under more normal environmental conditions. Therefore, this review has steered away from considering this work in any extensive way.

ROS for signalling is constrained by their chemistry irrespective of the system

ROS are highly reactive and in most cases, have a short lifetime, making them difficult to study. This is further compounded by the many different types of ROS, their preferred biological target within a cell, and conversions between different ROS species [3-7]. Reactive nitrogen species and other endogenously produced gases cross-react with many ROS [8], adding yet more complexity.

The ROS superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) arise as reductive intermediates between molecular oxygen (O_2) and

water [9]. The strategy for any (micro)aerobic living system is to minimise the formation of $OH^{\bullet}$, the most reactive chemical species in biological systems, which is achieved by non-enzymic and enzyme-catalysed scavenging of $O_2^{\bullet-}$ and H_2O_2 [10]. There also are no free transition metal ions to catalyse the reaction of these two ROS to produce $OH^{\bullet}$ in the Haber-Weiss reaction [10, 11]. The extreme reactivity of $OH^{\bullet}$ is thought to preclude it from any direct role in signalling [12].

$O_2^{\bullet-}$ has been implicated in signalling in bacteria. In *Escherichia coli* the soxR and soxS proteins are oxidised by $O_2^{\bullet-}$ to activate the expression of genes of the soxR/S regulon [13]. However, in studies on eukaryotic systems, attribution of this ROS as directly engaging in signalling has, to our knowledge, never been made. It is H_2O_2 which has received the most attention as a signalling molecule [14]. Relative to the other ROS, it is the most stable of them and while it is still a potent oxidant capable of oxidising cellular macromolecules, it is less reactive. These intrinsic chemical properties, its synthesis and accumulation in several subcellular locations, its potential mobility within and between cells and its rapid reduction by highly redundant enzymic antioxidant systems, make H_2O_2 an ideal signalling molecule [15,16].

A further ROS worthy of mention is singlet oxygen ($^1O_2$). This is different from the other ROS in that it has not gained electrons but is rather an excited state of ground state O_2 [17]. $^1O_2$ is highly reactive, so much so, a little like $OH^{\bullet}$, it is thought not to diffuse far from its site of production [18], although mobility of this ROS has been implied in one study in *Chlamydomonas* [19]. However, in plants, $^1O_2$ initiates

several signalling responses to HL and other stresses and is considered in more detail below.

SUBCELLULAR SOURCES OF ROS FOR SIGNALLING IN HL

Photoinhibition of photosynthesis and production of $^1O_2$ in chloroplasts

When leaves of plants grown at a certain light level are exposed to a higher light intensity this can exceed the extant capacity of photosynthesis to use the absorbed energy. Under these HL stress conditions, increased production of ROS occurs in different sub-cellular compartments [20]. Conversely any other abiotic or biotic stress that depresses photosynthesis will lower the threshold at which HL causes increased ROS production. For example, chilling in the light will lower the rates of the Calvin-Benson Cycle reactions of photosynthesis without substantially lowering the rate of light harvesting [21]. Drought stress brings about closure of stomata that restrict carbon dioxide diffusion into the leaf. This has the same effect as chilling *i.e.* creating an imbalance between the absorption of light energy and the plant's ability to use it productively [20,22]. However, if the imbalance between the absorption of light energy and its consumption by primary metabolism continues or gets worse, then Photosystem II (PS II) reaction centres sustain more oxidative damage than can be repaired. This irreparably inhibits photosynthesis leading to a decline in plant productivity [23]. Under such an imbalance, the level of $^1O_2$ at PS II reaction centres can rise substantially because of resonance transfer of light energy from excited triplet chlorophyll to ground state O_2 [17] and is regarded as the underlying cause of irreversible photoinhibition [23,24]. $^1O_2$ is primarily quenched by carotenoids and probably α-tocopherol [25] present in large amounts in the thylakoids. The reaction of carotenoids with $^1O_2$ can give rise to signalling molecules [26] (see below). More universally, is the reaction of $^1O_2$ with fatty acids, which give rise to a cocktail of lipid-

soluble peroxides and peroxy radicals [24, 27, 28], which can carry out signalling (see below).

Reduction of O_2 in chloroplasts, the dissipation of excitation energy and the potential for generating H_2O_2 for signalling.

Before oxidative damage to PSII becomes significant, absorbed light energy (more accurately termed excess excitation energy; EEE) is dissipated safely as heat through resonance transfer of the absorbed light energy from excited chlorophylls to xanthophyll carotenoids by non-photochemical quenching. The reader is referred to several excellent descriptions of this crucial component of plant photo-protection [20, 29, 30], which will not be further considered here. EEE is dissipated also by several diverse metabolic processes, collectively termed photochemical quenching (PQ), which act as electron sinks and thus prevent over-reduction of the photosynthetic electron transport (PET) chain [20]. PQ processes so far identified include stimulation of primary (photosynthetic) metabolism [31, 32] and so-called "wasteful" reactions such as the Water-Water Cycle (WWC), the activity of plastid terminal oxidases (PTOX) and in C_3 plants, photorespiration (see below).

Primarily based on studies of algae, a key PQ process was identified as the WWC [33 - 35], which in response to HL, brings about the reduction of O_2, derived from the splitting of water in PS II, back to water at the acceptor side of PS I and consumes reducing equivalents in the process (Figure 1).

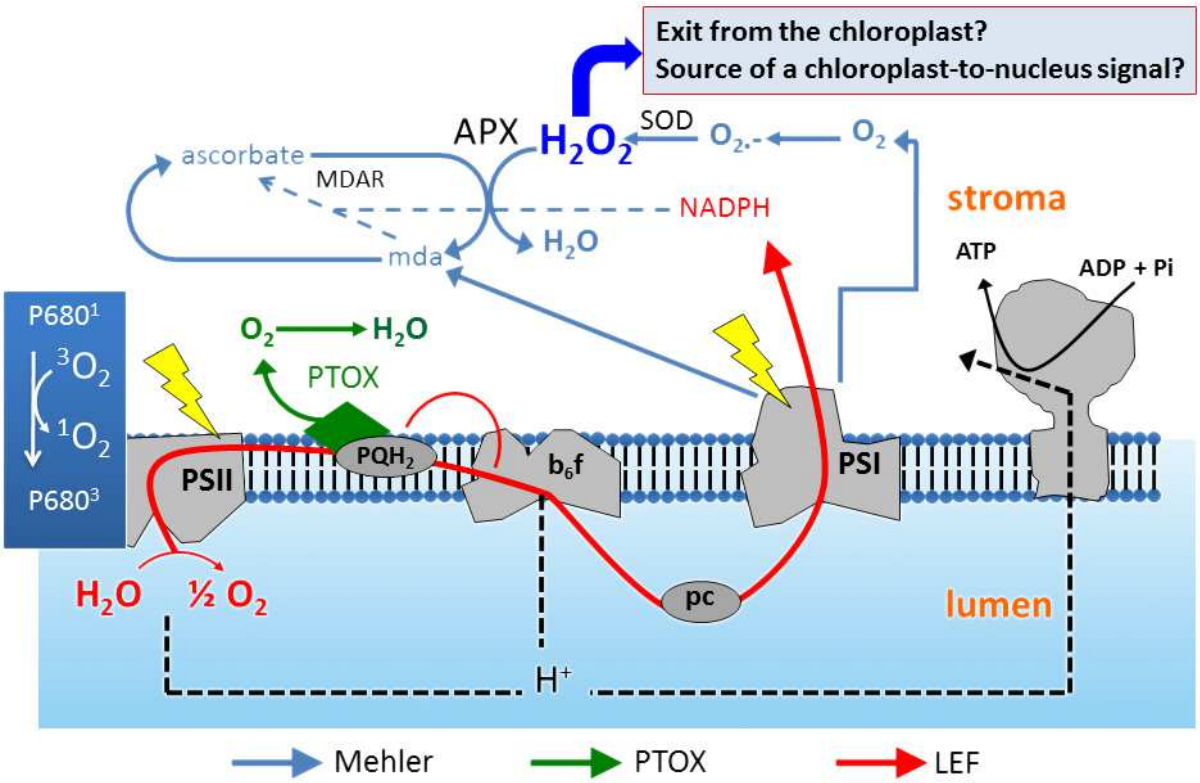


Fig. 1 Photo-reduction of O_2 and generation of ROS in higher plant chloroplasts. ADP + Pi adenosine diphosphate and inorganic phosphate; APX2 ASCORBATE PEROXIDASE2; ATP adenosine triphosphate; b6f Cytochrome b6f complex; H2O water; H2O2 hydrogen peroxide; LEF linear electron flow; MDA monodehydroascorbate radical; MDAR monodehydroascorbate reductase; NADPH Nicotinamide adenine dinucleotide phosphate; O2 molecular oxygen; P680 primary oxidant of PSII; PC plastocyanin; PQH2 plastoquinol; PSI photosystem I; PSII photosystem II; PTOX plastid terminal oxidase; SOD superoxide dismutase.

The reduction of O_2 to $O_2^{\cdot-}$ is the Mehler reaction and gives rise to H_2O_2 via the action of superoxide dismutases (SODs; Figure 1). In algae and cyanobacteria, there is no doubt that the WWC is a major means of dissipating excitation energy. For example, it accounts for up to 49% of total electron flux in diatoms [36] and occurs as a significant activity throughout the Chlorophyte lineage up to and

including the Gymnosperms [37]. The high activity of WWC in these organisms does not necessarily mean an accompanying high level of $O_2^{\cdot-}$ and H_2O_2. This is because of the flavodiiron (FLV) proteins, which as O_2 reductases, catalyse the rapid reduction of O_2 directly to water minimising intermediate ROS formation. FLVs are prokaryotic in origin but again can be found all the way up to the Gymnosperms, but importantly, not in the Angiosperms [34, 35, 38, 39].

In Angiosperms, the activity of WWCs and Mehler reaction is low, with measures of PET-driven O_2 reduction ranging from zero to a maximum of 4.4% of total linear electron flux in bean leaves under HL at their CO_2 compensation point (*i.e.* where the CO_2 fixed by photosynthesis equals that produced by respiration) in the absence of photorespiration [37, 40, 41]. Nevertheless, $O_2^{\cdot-}$ can be detected in leaves in response to HL and other stresses [41 - 43]. More commonly, there have been many reports of photosynthesis-dependent HL-induced increases in foliar H_2O_2 levels, which in Arabidopsis shows preferential accumulation in leaf bundle sheath cell chloroplasts [41, 44 - 46]. Recently the use of HyPer, a GFP-based specific H_2O_2 sensor, demonstrated the dynamics of production in *Nicotiana benthamiana* chloroplasts' stroma, which begins at 10 mins after the onset of HL, is PET-dependent and is both an initiator and likely transducer in chloroplast-nucleus signalling (see below) [47]. The use of HyPer can be challenging due to its pH sensitivity and a tendency to be silenced in stable transgenic plants. Nevertheless, using this or similar genetically-encoded biosensor proteins, it is now theoretically possible to determine the concentration of H_2O_2 in the chloroplast stroma. Therefore, there is now the prospect of determining the rates of change in H_2O_2 concentrations on the stromal side of the thylakoids and matching this against both the overall rates of PET and O_2 photo-reduction (including chlororespiration). Would we see any

agreement in these values compared with the low rates of O_2 reduction determined under the same conditions and therefore, is this production of H_2O_2 in chloroplasts a consequence of Mehler reaction activity? This question is not easy to answer and it is not even clear if the Mehler reaction in Angiosperms is driven by one or more specific proteins.

It is possible to demonstrate *in vitro* that the reaction of ascorbate with $^1O_2$ results in H_2O_2 at a 1:1 stoichiometry under physiologically relevant conditions and therefore could be considered an alternate source in chloroplasts of this ROS for signalling [4]. However, exposure to HL of leaves treated with the PET inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which causes a strong increase in $^1O_2$ levels [48, 49] does not cause any increase in H_2O_2 in the chloroplast stroma [47]. This excludes the likelihood that $^1O_2$ would be the source of H_2O_2 for signaling in the chloroplast in response to HL.

The involvement of PTOX rather than FLVs can provide another version of the WWC. There is considerable evidence that in algae and higher plants that PTOX acts as an alternative electron sink and therefore contributes to PQ [34, 50 - 52]. However, of relevance to this review is the question of whether PTOX can produce $O_2^{\cdot-}$, which would dismutate to H_2O_2 that could be co-opted for signalling. This is a controversial question to address. The debate is still on-going with the argument that PTOX conducts side reactions that generate these ROS [35, 51- 53]. This is based on studies of the enzyme *in vitro* and thylakoid preparations from transgenic tobacco over-expressing Arabidopsis PTOX. The PTOX over-expressing plants do produce $O_2^{\cdot-}$and H_2O_2 but it is not clear that *in vivo* the recombinant PTOX is directly responsible for this. The counter argument is that in comparisons of plant species of the same genus, or different genotypes of the same species that harbour differing

PTOX activity do not show proportional changes in ROS levels [50, 54, 55]. On the contrary, PTOX activity has been associated with reduced levels of H_2O_2 because it competes for reducing equivalents with the Mehler reaction [56]. Further, at least one *in vitro* study has ruled out ROS production by PTOX [57]. Consequently, detailed reviews on PTOX do not raise this as a possibility [52].

Are peroxisomes sources of ROS for HL signalling?

In C_3 plants exposed to HL, photorespiration accounts for a substantial amount of Rubisco activity in competition with photosynthesis. Photorespiration is an intrinsic activity of Rubisco and is the catalysed oxidation of ribulose bisphosphate with O_2 rather than CO_2, generating phosphoglycolate rather than 3-phosphoglycerate [58]. Photorespiration in the chloroplast is not a source of ROS but the photorespiratory cycle, which is used to return glycolate to primary metabolism, is a major source of H_2O_2 in peroxisomes [59]. The value or otherwise of photorespiration to plant/crop productivity is the subject of considerable debate [60 -62], but it is a highly significant PQ process.

The further oxidation of glycolate to glyoxylate is catalysed by glycolate oxidase [61]. In doing so, this reaction generates H_2O_2 in peroxisomes, that even with a strong antioxidant system, has been estimated to produce steady state levels in the order of 10 µM [16]. To deal with this amount of H_2O_2, peroxisomes have a highly developed enzymic and non-enzymic antioxidant system, the most prominent and specific feature being the large amounts of the enzyme catalase [59]. Therefore, while photorespiration under HL can bring about a substantial increase in H_2O_2 levels and the process may be of value in dissipating EEE, the question is does this source of H_2O_2 gives rise to specific signalling events under these conditions? There is an extensive body of work using both tobacco and Arabidopsis plants deficient in

peroxisomal catalase activity [63 - 65]. Such plants often show HL sensitive phenotypes and altered expression of at least several hundred genes, many being annotated as stress responsive or defensive and their regulation mediated by stress hormones such as salicylic acid [63, 65 - 68]. This could be taken to mean that H_2O_2 from peroxisomes act to initiate and/or transduce signals to the nucleus in response to HL. However, it is difficult to see how the study of catalase-deficient mutants allows an extrapolation to the wild type plant under HL. In this respect, what may be of significance is that such plants display an oxidative stress phenotype analogous to plants undergoing a non-limited hypersensitive immune response displayed by lesion mimic mutants, plants suffering acute ozone fumigation, exposure to the herbicide paraquat or an induced increase in $^1O_2$ production [43, 69, 70]. To some extent all these observations on disparate experimental systems suggest that there is a common signalling response to oxidative stress that may be associated with the triggering of programmed cell death (PCD) [70]. This would be manifest in wild type plants during an immune response to some pathogens or extreme abiotic stress such as a very HL provoking extensive photoinhibition [71, 72]. Unfortunately, evidence is lacking that under such circumstances peroxisome-sourced H_2O_2 plays a signalling role. We suggest those unusual situations for the plant such as a permanent and debilitating loss of catalase activity lead to oxidative damage, which provokes PCD as a default response [70].

More recently, experimental evidence has shown that only H_2O_2 sourced from chloroplasts initiates HL signalling and one corollary of this was to exclude it from other major cellular sources such as peroxisomes [47]. It should be emphasised that this conclusion does not exclude peroxisome-sourced H_2O_2 being a source for signalling in wild type plants under other stresses, but in HL it could be that

increased H_2O_2 in peroxisomes is scavenged effectively and is not able to initiate signalling.

Are mitochondria sources of ROS for HL signalling?

The mitochondrial respiratory electron transport chain (mtETC) is a source of $O_2^{\cdot-}$ and its consequent dismutation to H_2O_2 (see above) occurs during normal respiration. Over-reduction of the mtETC leads to a substantial increase in these ROS, occurring when cells are treated with inhibitors of respiration or where ADP supply becomes limited. In mammalian cells, increased ROS production has been associated with pathologies that induce mitochondrial dysfunction thus triggering apoptosis. The reader is referred to an excellent review [73] for a detailed description of how ROS arise from different points along the mETC and how these initiate signalling. In plants, there is good evidence for the mtETC being a source of $O_2^{\cdot-}$ and H_2O_2 and considerable genetic and biochemical evidence, that as in animals, mitochondria in plant cells do play roles in PCD. The reader is referred to a recent paper [12] regarding a critical review of this subject but it should be noted that as with dysfunctional peroxisomes, ROS production by dysfunctional mitochondria produces changes in the transcriptome reminiscent of the many oxidative stress-associated signalling events mentioned in the previous section [74].

Mitochondria are important in HL responses of wild type plants through increased respiration contributing to PQ [75,76]. In addition, HL also induces the expression of nuclear genes coding for ALTERNATIVE OXIDASE (AOX) [76]. AOX isoforms lower the energy efficiency of mitochondrial electron transport by diverting it from ubiquinone to O_2 generating water without any intermediate ROS production [77]. In addition, uncoupling proteins cause leakage of protons across membranes, which prevent over-reduction of mtETC components, thus lowering the opportunity

for O_2 reduction to promote ROS formation. Therefore, under HL conditions the mtETC undergoes changes that seem to minimise ROS production but increase its contribution to PQ. However, while there is evidence of retrograde signalling from mitochondria operating in HL, such as the often-observed induction of AOX gene expression and increased AOX protein levels [74, 78], a critical role for ROS in this process is absent.

In mammalian cells, the relationship of ROS and mitochondria is important since all evidence points to them having a crucial role in retrograde signalling [73, 79-84]. Exposure to light from lasers in mammalian cells cause mitochondrial membranes to become depolarised at excessive ROS concentrations [73, 85 - 87] and may be destroyed in a ROS burst by prolonged opening of mitochondrial permeability transition pores [88 - 90]. This mitochondrial permeability transition can induce DNA fragmentation [91] and apoptosis [92]. The mitochondrial membrane contains key regulators (cytochrome c, the Bcl-2 family of proteins) of the balance between apoptosis and survival. Even at low irradiance, mitochondria-derived ROS may lead to cell death [93]. One possible reason is that mitochondria are rich in chromophores that are photosensitive in the UV and visible wavelength range, such as heme proteins, flavoproteins, and NADH or NADPH [94 - 96]. This is compounded by the complex interaction of mitochondria with the endoplasmic reticulum (ER) [97, 98].

Perhaps one of the most intriguing questions that remains to be investigated is whether mitochondria and peroxisomes fulfil different signalling functions in cells with or without chloroplasts. We believe that invaluable information can be gained from comparing redox biology across species and kingdoms to answer this question.

Extracellular, cytosolic and plasma membrane sources of ROS for HL signalling

There are many potential sources of $O_2^{\cdot-}$ and H_2O_2 arising from specific enzyme reactions located in the cytosol, plasma membrane and cell wall. In the cell wall these reactions, such as from anionic peroxidases [99] and berberine bridge reticuline oxidases [100, 101], have been suggested to act as sources of H_2O_2 for signalling, although their primary purpose is likely to provide it for oxidative cross-linking of lignin into the cell wall matrix [102]. In any case, no link to HL responses has been reported. In contrast, the plasma membrane-associated NADPH oxidases have been strongly implicated in signalling, initially as part of the hypersensitive response and then in the abscisic acid (ABA) -triggered closure of stomata [103]. In Arabidopsis bundle sheath cells, the HL-mediated induction of *ASCORBATE PEROXIDASE2* (*APX2*) is controlled in part by a ABA-signalling pathway consisting of a core protein phosphatase 2A ABSCISIC INSENSITIVE1 (ABI1)/ ABI2 / SUCROSE NON-FERMENTING RELATED PROTEIN KINASE2.6 (SnRK2.6) regulatory module [45, 104, 105]. The RESPIRATORY BURST (NADPH) OXIDASEF (RBOHF) whose production of $O_2^{\cdot-}$ is stimulated by ABA, was also shown to be required for induction of *APX2* expression [45]. It was suggested that the resulting H_2O_2 is part of a feedback loop that inhibits redox sensitive ABI2 activity thereby stimulating SnRK2-mediated ABA control of *APX2* expression [45, 106]. Whereas mutations in this signalling pathway affect the ability of bundle sheath cells to respond to HL, they have no impact at the whole leaf level [45]. However, an enhanced capability of bundle sheath cells to produce $O_2^{\cdot-}$ and H_2O_2 [42, 106] may be important in the systemic transmission of a localised intense HL exposure. Extracellular H_2O_2 was shown to be a component in the long-range transmission of

signals that establishes a degree of acclimation to HL in previously un-exposed parts of the plant [107, 108]. This vectored systemic signal has been shown to involve ABA, RBOHD and Ca^{2+} in a H_2O_2- and voltage-mediated auto-propagating signal that passes rapidly from cell-to-cell [109, 110]. The HL exposures used were high and certainly produce strong photoinhibition, cell death manifested as bleaching of leaf tissues and gene expression signatures associated with PCD [107, 108]. Yet no involvement of $^1O_2$ signalling has been implicated in this systemic signalling. In the HL-exposed cells this would certainly be the case and it would be of considerable interest to determine if this ROS appeared during the propagation of a ROS wave. Under more moderate localised HL exposures there is also some evidence of a systemic stimulation of antioxidant defence gene expression and production of extracellular H_2O_2 [46, 106]. Therefore, we speculate that signalling for a wide range of changes in PPFD, which may involve different ROS, nevertheless possess a systemic component linked to each specific intracellular signalling network.

$^1O_2$ AND H_2O_2 AS INITIATORS AND TRANSDUCERS OF SIGNALS FROM CHLOROPLASTS TO THE NUCLEUS DURING HL

The high reactivity of $^1O_2$ (see above) means that it is likely to initiate rather than directly transduce signalling pathway(s) out of the chloroplast in response to HL. Within PS II reaction centres, $^1O_2$ is quenched rapidly by carotenoids and one of the resulting oxidation products, ß-cyclocitral, could act as the transducer of a HL $^1O_2$-initiated signalling pathway [26]. Treatment of low-light grown plants with ß-cyclocitral altered the expression of >1,000 genes and increased their tolerance to photooxidative stress. Importantly, *ca.* 25% were in common with the transcriptome profile of the $^1O_2$ *flu* Arabidopsis mutant reinforcing the notion that ß-cyclocitral transduces a $^1O_2$ signal. While these observations are convincing there is yet no

further details of how (or if) ß-cyclocitral exits the chloroplast, whether it undergoes further metabolism or what its “receptor” could be to further transduce the signal. ß-cyclocitral is a reactive electrophilic species and therefore could react with electron donors such as sulfhydryl groups in proteins [26]. This implied involvement of redox-active proteins is attractive as this would allow an $^1O_2$ signal to a converge into a generalised oxidative stress signalling network originating from several distinct sources and environmental challenges.

There is strong evidence for a second $^1O_2$ pathway, which in our view, is, to date, the most completely defined HL-initiated ROS-mediated retrograde signalling pathway. Exposure of wild type Arabidopsis plants to HL that promotes photoinhibition produces distinctive peroxidised polyunsaturated fatty acids (PUFAs) [24]. *In vitro* reduction of lipid extracts from HL-exposed leaves generated two hydroxy-PUFA isomers, 10- and 15-HOTE (HOTE stands for 10-hydroxy-8,12,15(E,Z,Z)octadecatrienoic acid) that are derived from their peroxide equivalents. These arise from the reaction of $^1O_2$ with linolenic acid. Therefore, the possibility arose that lipid peroxidation arising from $^1O_2$ in PSII reaction centres could transduce a retrograde signal. This was investigated further using the *chlorina1* (*chl1*) Arabidopsis mutant, which is extremely sensitive to photooxidative stress in HL because of impaired NPQ and a loss of structural integrity of PS II reaction centres [111]. This was attributed to increased $^1O_2$ leading to increased detection of 10- and 15-HOTEs indicative of increased lipid peroxidation [111]. Transcript profiling revealed a strong signature for signalling associated with the stress hormone jasmonic acid (JA), which is synthesised from PUFA hydroperoxides [112]. Increased production of JA and JA-mediated signalling had been observed in the $^1O_2$-generating *flu* mutant [113, 114]. A double mutant, *chl1/ delayed dehiscence2*

(*dde2*) was more resistant to photo-oxidative stress in showing less tissue bleaching and lipid peroxidation than *chl1* [111]. *Dde2* is defective in allene oxide synthase and cannot further metabolise 13-hydroperoxy linolenic acid and consequently cannot synthesise JA. Therefore, JA could be part of a $^1O_2$ signalling pathway that triggers a HL-associated PCD in *chl1*. Moderate HL exposure of *chl1*, leading to some increase in $^1O_2$ production, does prime the plant to be more tolerant of subsequent higher light intensities and interestingly, JA treatment of these plants blocks this subsequent acclimation pushing the plant into a PCD response [111]. In seedlings, it has been argued that moderate levels of $^1O_2$ production do indeed promote acclimation to HL but this is mediated by the *EXECUTER1* (*EX1*) and *EX2*-dependent pathways identified from a screen for reversion mutants of *flu1* [48] [115] [116] and is distinct from the $^1O_2$- JA-dependent pathway identified in *chl1*[111]. Equally the $^1O_2$ initiated *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*)- and salicylic acid-mediated signalling pathway identified as operative in *flu1* appears to play no role in HL responses [69, 111, 113, 117]

In summary, the work on *chl1* combined with knowledge on JA biosynthesis and downstream signalling means that this lipid peroxidation-mediated pathway can describe initiation of signalling by $^1O_2$, signal transduction via PUFA peroxides leading to stimulation of JA biosynthesis, part of which is extra-plastidial thus allowing a signal to exit the chloroplast [112]. The receptors for JA signalling (JAZ proteins) which in the absence of JA, are repressors of transcription have been identified [112] and there are clear and specific changes in the expression of a panel of genes common to both $^1O_2$-mediated HL responses and JA responses. Taking these studies together, a detailed HL- and $^1O_2$-driven retrograde signalling pathway

clearly could be outlined (Fig. 2).

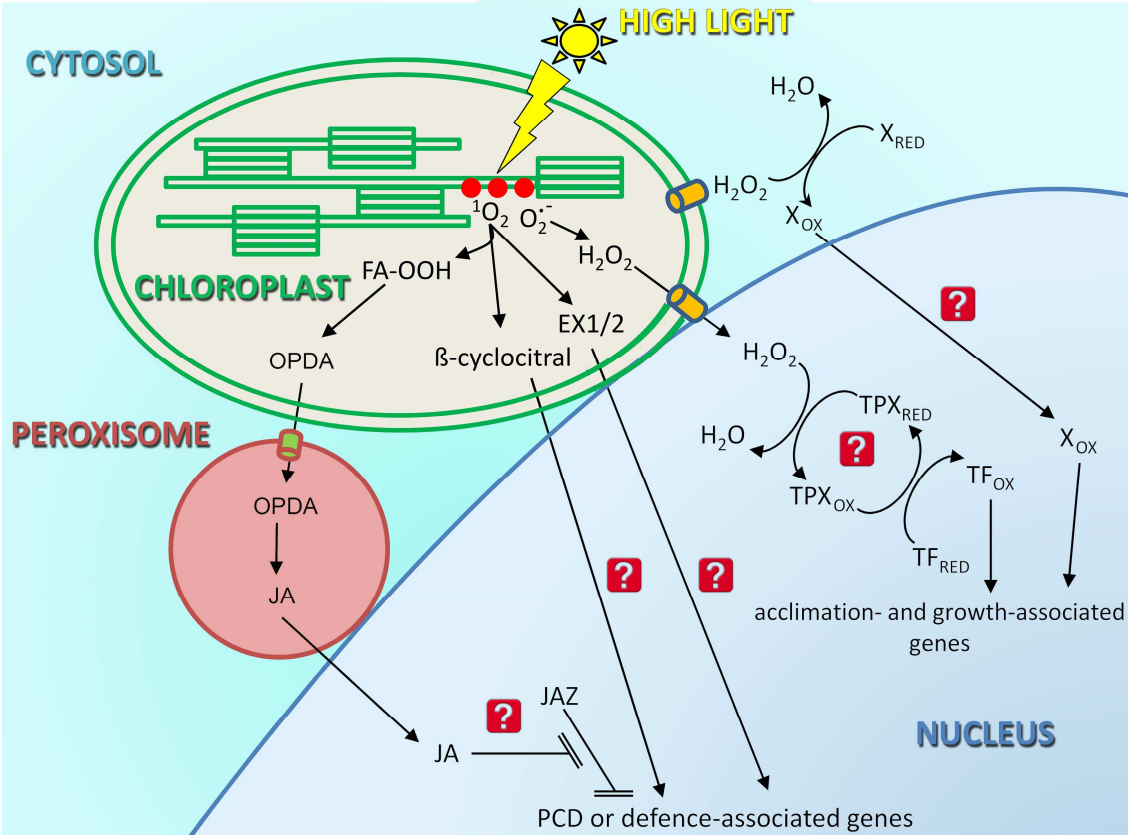


Fig.2. Proposed chloroplast-to-nucleus retrograde signalling routes initiated and/or transduced by $^1O_2$ and H_2O_2. Question marks (?) are presumptive steps currently lacking experimental evidence. Cylinders coloured yellow and green depict aquaporins (AQPs) and a peroxisomal ABC transporter respectively. OPDA, 12-oxophytodienoic acid; JA, jasmonic acid; JAZ; JA receptor.

The operation of this proposed JA-dependent pathway and other stress hormone pathways is consistent with, and is an extension of, an emerging important role for ROS, chloroplasts and photosynthesis in the immunity of plants to pathogens [72, 118].

Retrograde signalling derived from primary metabolism. Do ROS have a role?

In recent years, considerable evidence has accumulated that chloroplast retrograde signalling need not involve ROS at all. Intermediate compounds in primary metabolic networks have proved to be promising HL retrograde signalling molecules, which in some cases includes evidence that such molecules transduce the signal out of the chloroplast. Prominent among these molecules are 3' phosphoadenosine 5' phosphate (PAP), methyl erythritol cyclodiphosphate (MEcPP), and triose phosphate [119 - 121]. Metabolite profiling of HL-exposed plants or those with altered chloroplast redox states suggest that many more primary and intermediary metabolites could act as signalling molecules [122, 123].

The question is, can ROS influence the levels of these metabolite retrograde signalling molecules? If yes, then ROS could initiate or participate in retrograde signalling through the metabolism of these molecules. PAP is degraded by the enzyme SAL1 and consequently negatively regulates responses to HL [119]. SAL1 has four redox-sensitive Cys residues that, when oxidised or mutated to Ala, reduce the specific activity of the enzyme by 50% [124]. The oxidising conditions inside the chloroplasts needed to drive this response produce changes in stromal glutathione redox state, which means this system may activate only under photo-oxidative stress [124]. Nevertheless, this clearly shows how $O_2^{\cdot -}$ and H_2O_2 could influence a metabolite-directed retrograde signalling pathway. In contrast, signalling mediated by triose phosphates occurs more rapidly than that mediated by H_2O_2 [121], perhaps ruling out a role for signalling in this pathway.

H_2O_2 as HL retrograde signalling transducer and the containment hypothesis.

In animal cells, increased antioxidant content or ROS scavenging capacity is protective and enhances tolerance to oxidative stress [125, 126]. In plants subjected to HL and photooxidative stress, this is not the case. Paradoxically, enhanced levels

of the thiol antioxidant glutathione in chloroplasts, increases plants' susceptibility to photoinhibition and triggers a PCD-like response [127, 128]. Transiently increasing ascorbate peroxidase (APX) activity in the chloroplast stroma or infiltration of reduced glutathione inhibits HL-induction of nuclear gene expression [47, 129]. Conversely, treating leaf tissue with 1-10 mM H_2O_2, a level that would cause oxidative stress in animal tissues, improves tolerance to photooxidative stress, decreases susceptibility to photoinhibition and induces the expression of genes coding for a wide range of functions [49, 107, 129 - 132]. Removal of multiple H_2O_2 scavenging enzymes can lead to increased oxidative stress tolerance compared with the loss of a single gene [133 - 135]. These observations spanning two decades indicate that H_2O_2 has a role in inducing protective or acclimation responses to HL.

The chemical stability of H_2O_2 compared with other ROS suggests that it could be a mobile signalling molecule transducing signals out of the chloroplast [136]. However, the cytosol contains a substantive H_2O_2 scavenging capacity, which is further enhanced upon exposure to HL [46, 129, 131, 137 - 139]. Therefore, it was hypothesised that under moderate HL conditions, the H_2O_2 signal would be converted to another molecule which would be unaffected by the reducing environment of the cytosol. This was termed the containment hypothesis, which postulated that for HL acclimation this was necessary. Where HL triggered PCD, this was proposed to be because H_2O_2 had accumulated to sufficient levels to diffuse out of chloroplasts, exceed the capacity of cytosol antioxidant scavenging and trigger oxidative stress in other parts of the cell [139]. Isolated chloroplasts exposed to HL secrete H_2O_2 into their medium [140], which supported this hypothesis. However, spatial, temporal and non-invasive quantitative imaging of H_2O_2 in the cytosol [47] clearly showed that the containment hypothesis was only partly correct. Under

moderate HL conditions, H_2O_2 was observed to accumulate in the cytosol but was confined to microdomains adjacent to chloroplasts. This is similar to H_2O_2 micro- or nanodomains as sources of signalling observed in animal cells [141 - 144]. Therefore, still in keeping with the containment hypothesis, H_2O_2 would not be able to traverse the cytosol to deliver a chloroplast-sourced HL signal to the nucleus. The observation of chloroplast-associated cytosolic H_2O_2 microdomains implies that if there is onward signalling, the H_2O_2 would have to interact with the next step in the transduction pathway in the microdomain, imposing a spatial constraint on the process [144]. For example, in HL, the conversion of the Heat Shock Transcription Factor A1d (HSFA1d) from its inactive monomeric form to an active trimer may require H_2O_2 in the cytosol [138]. Therefore, this spatial constraint would require HSFA1d or an intervening transducing protein to be present in the microdomain at the right time. How this could be achieved is not known.

Spatial components to ROS signalling from organelles to the nucleus – a means of increasing specificity.

Under normal growth conditions, in different plant tissues and algal species, a proportion of chloroplasts in cells have been shown to appress to nuclei with some studies reporting ER interposed between them [144 - 149]. In Arabidopsis and *Nicotiana benthamiana* (*N. benthamiana*) epidermal cells a proportion of leucoplasts and chloroplasts respectively are associated with the nucleus [47,150]. Under HL conditions, it was established that in *N. benthamiana* epidermal cells, chloroplast-sourced H_2O_2 was rapidly directly transferred to the nucleus without traversing the cytosol and was linked to the induction of a HL-responsive gene expression [47]. This provided evidence that the spatial association of chloroplasts with the nucleus is critical in providing signalling specificity. In epidermal cells of Arabidopsis, the

nucleus has been observed to move away from incident blue light [152, 153]. However, in the *chloroplast unusual position1* (*chup1*) mutant, this fails to occur. This is because in this cell type, the nucleus depends upon its associated leucoplasts to move in response to incident light [152, 153]. The CHUP1 protein is expressed in photosynthetic tissues and is in the chloroplast envelope. CHUP1, *via* F actin filaments, provides linkage to the nuclear envelope [151, 153]. The need to move the nucleus in response to incident irradiation was suggested to be a protection against UV damage [152, 153]. However, this linkage could also be required to achieve retrograde signalling to coordinate plastid and nuclear responses to HL. In strong support of this argument, is that *chup1* suffers more photoinhibition under HL than its wild type counterpart [152]. In the context of spatial retrograde signalling, this *chup1* HL phenotype would be because of a disruption in the direct transmission of retrograde signals from chloroplasts to nuclei.

An additional and intriguing point is that stromal APX isoforms contain a 16-residue insertion which is not present in cytosolic or glyoxysomal isoforms. This insertion renders these isoforms more sensitive to H_2O_2 inhibition than cytosolic isoforms [153]. It has been speculated that the increased sensitivity of stromal APX to H_2O_2 has evolved to allow a threshold to be set that would trigger chloroplast-to-nucleus signalling [47, 154].

The transfer of H_2O_2 to the nucleus must include it crossing the chloroplast double membrane. It has been suggested that H_2O_2 can passively diffuse across membranes using channels formed by aquaporins (AQPs) [155]. Water and H_2O_2 share similar physicochemical properties such as their dipole moment and surface charge distribution, which allow them to use the same AQPs [155, 156]. There are at least 4 AQPs in the chloroplast inner membrane [157]. However, one or more of

these will be CO_2 channels that would not allow passage of water [158]. How much further the identification of an H_2O_2-accepting AQP should be taken is a moot point, since the disruption of AQP expression (e.g. by reverse genetic approaches) would disrupt water movement as well, leading to difficulties over the interpretation of experimental outcomes. Transit of H_2O_2 across the chloroplast outer membrane would be most likely occur passively through porins [159, 160], although to our knowledge no evidence has been presented to support this statement.

Under conditions associated with PCD, such as in the innate immune response and when PET is perturbed then hollow tube-like structures termed stromules have been observed connecting chloroplasts to nuclei [161 - 163]. PCD is associated with rendered the stroma being much more oxidising leading to increased ROS production [162, 163]. There is evidence that proteins and H_2O_2 can transfer across to the nucleus though stromules and therefore the potential for retrograde signalling is clear [162, 164]. However, under moderate HL conditions which do not produce such drastic changes in the redox environment of the stroma, stromules were not observed [47]. One consideration could be that the HL exposures were no longer than one hour, while conditions which caused stromule formation took several hours to have an effect. Therefore, it is possible that direct chloroplast-nuclei associations transition to stromule-mediated connections as stress increases in severity and duration. This perhaps would allow a wider range of signalling molecules (including proteins) to be transferred to the nucleus as stress increases. Nevertheless, given that we observed no stromules under HL, we will not consider stromules further in this review. In other published time-lapse movies, stromules did not grow in the direction of the nucleus, so their role in plastid-nuclear communication may altogether be more indirect [165].

H_2O_2: The signalling messenger to the nucleus

In HL-exposed cells, chloroplast-sourced H_2O_2 accumulates in the nucleus and triggers gene expression, thus initiating cellular responses to the changed light conditions [47]. In plants, very little is known about how the H_2O_2, once arrived in the nucleus, transduces the HL signal. At least two broad possibilities are discernible for how H_2O_2 may transduce the HL signal. H_2O_2 could either oxidise multiple redox-sensitive regulatory proteins directly or oxidise an intermediate messenger(s) to relay oxidising equivalents to a target regulatory protein. For example, the plant transcription factors from the AP2/EREBP, bZIP TGA and HSF families have been proposed to undergo redox modifications and have been implicated in transcriptional responses to HL [138, 166 - 172].

The direct oxidation by H_2O_2 of regulatory proteins appears to be the norm in prokaryotes, exemplified by the induction of gene expression in *Escherichia coli* and *Bacillus subtilis* by the transcription factor oxyR and perR, respectively [173 - 175]. However, in eukaryotes this is generally considered not to be the way in which H_2O_2 transduces its signals. Partly this is because of a multiplicity of H_2O_2-responsive networks and processes, begging the question of how specificity might be achieved, especially in the face of observed differential responses along intra- and intercellular gradients of H_2O_2 [144, 176, 177]. Protein kinases, protein phosphatases, proteasome components and transcription factors have been shown to possess redox-sensitive cysteine thiols from *in vitro* assays, including from plants [175]. Unfortunately, not many of these studies provided kinetic information that would allow an assessment of the likelihood of a direct oxidation of the protein by H_2O_2 *in vivo* [175]. Where kinetic measurements are available, rate constants of 10^1 -10^2 $M^{-1}s^{-1}$ for reduction of H_2O_2 are typical, approximately 3-5 orders of magnitude lower

than antioxidant enzymes [175]. Furthermore, the cellular concentrations of antioxidant and ROS-scavenging enzymes are much greater than that of regulatory proteins and would win the competition for reacting with H_2O_2 [178]. For these reasons, direct modulation by H_2O_2 of regulatory proteins is considered unlikely. However, this assumption has been questioned [175]. Modelling of reactions of a redox-sensitive target protein with H_2O_2 by varying a range of kinetic parameters allowed a determination of the concentration of H_2O_2 required to achieve response times of 30s, 5 min and 60 min. If a slow response is required, measured in tens of minutes, then physiologically relevant micromolar concentrations of H_2O_2 could achieve some oxidation of a target protein, even in the face of a substantial cellular H_2O_2 scavenging capacity. *In vitro*, the Arabidopsis protein phosphatase ABI2 is 50% inhibited by 50µM H_2O_2 in the presence of 1-10 mM reduced glutathione [179] suggesting it could be a direct interactor with H_2O_2. However, even for this rare example from plants, ABI2 *in vivo* nevertheless may have a redox relay intermediary, GLUTATHIONE PEROXIDASE3 (GPX3), between it and H_2O_2 [180]. Clearly, the extent and duration of a H_2O_2 signal become critical factors to determine the likelihood of direct interactions between target regulatory proteins and H_2O_2. The availability of quantitative *in vivo* H_2O_2 biosensors for plants [47, 181- 183] means a start can be made on answering this critical question.

Redox relay signalling: A guide to future research on HL and H_2O_2 signalling?

The alternative to direct oxidation by H_2O_2 of target regulatory proteins is that one or more intermediate proteins transfer oxidising equivalents in a series of linked redox relay reactions. The first description of such a system and still the paradigm to draw on is the yeast ORP1 – YAP1 module. This is where the induction of YAP1-mediated transcriptional responses to H_2O_2 is mediated by the transfer of oxidising equivalents

to the transcription factor YAP1 *via* transiently oxidised ORP1 [5, 178, 184, 185]. The key features of this system are that it is a 1-step relay and that ORP1 is a thiol peroxidase, hence its alternative name GLUTATHIONE PEROXIDASE3 (GPX3). This latter point is particularly important because thiol peroxidases, which include the GPXs and the peroxiredoxins (PRXs) are, in yeast, animal and plant cells, among the most abundant and potent of H_2O_2 and organic peroxide scavengers [178, 186 - 189]. So how is it that one of their number, ORP1/GPX3, rather than carry out its normal catalytic role can instead also act as a carrier of oxidising equivalents? For this redox relay event to work, a second adaptor protein, YBP1, is required to bring together YAP1 and ORP1/GPX3. When these two proteins are in a complex with YBP1, electron transfer from H_2O_2 to YAP1 relayed via ORP1 is preferred over the latter's peroxide scavenging role [190]. In addition, ORP1 is a scavenger of organic hydroperoxides but when complexed with YAP1 and YBP1 is definitely only a H_2O_2 sensor, implying that the complex also alters the substrate specificity of ORP1 [191].

Most, but not all, thiol peroxidases have a pair of catalytic redox active cysteine residues, the peroxidatic cysteine (CysP) and the resolving cysteine (CysR) [186, 192, 193]. In a "ping-pong" mechanism the transient formation of the sulfenic acid form of CysP (CysP-OH) occurs because of electron transfer to its substrate peroxide. The restoration of CysP to its thiol form completes the catalytic cycle [185]. This requires CysR and reducing equivalents, which would be from reduced glutathione (GSH), thioredoxin or glutaredoxin. Changes in the structure of thiol peroxidases during this catalytic cycle occur such that CysP-OH becomes briefly surface exposed [185, 193], providing an opportunity for electron transfer from an interacting protein to occur. More recently, attention has switched to the PRX thiol peroxidases as redox relay proteins. The most compelling evidence shows that the

only deletion of all 5 PRX and 3 GPX genes in yeast, while still allowing a viable cell, completely blocks all transcriptional responses to exogenous H_2O_2 [194]. This clearly establishes the PRXs as well as GPXs as transducers of a H_2O_2 signal to what must be a multiplicity of target proteins. All PRXs have been shown to be able to transfer oxidising equivalents to redox(ro)GFP2 *in vivo* when tethered to it by a polypeptide linker and oxidised roGFP2 is then reduced, most likely, by glutaredoxin [178, 181, 192]. Deletion of the CysR such as in the yeast TSA1 PRX does not impede its ability to act in a redox relay and the reduction-oxidation cycle of the TSA1 and roGFP2 moieties does not become blocked because of the cellular capacity to regenerate reduced roGFP2 [192]. This means that the rarer 1-CysPRX isoforms, which harbour the equivalent of only CysP [186, 195] could be involved in redox relay signalling.

Various approaches linked to proteomics and structural bioinformatics have shown that there are several hundred proteins with redox-active cysteine thiols that would be capable of interacting with other proteins [196 - 199]. While some of these approaches have been questioned [178], nevertheless evidence has been presented pointing to several endogenous 1-step redox relay signaling pathways in bakers' and fission yeast as well as more recently in animal cells [178, 185, 200]. Interestingly, PRXs in a number of organisms, including plants and algae, also display a circadian rhythm for the cellular levels of their oxidised and hyper-oxidised forms paralleling rhythms of their substrate peroxides [201]. However, this might also imply that there are regular periodic changes in the redox state and consequent activity of PRX-target proteins which, in turn, promote or dampen circadian influences on some cellular processes.

In plants, as in other eukaryotes, there are multiple isoforms of GPXs and PRXs that display considerable genetic redundancy. For example, in Arabidopsis, there are 12 PRX and 8 GPX isoforms [186]. Under HL conditions, the delivery of H_2O_2 directly to the nucleus and the possibility of H_2O_2 microdomains on the cytosol side of chloroplasts [47] would mean that considerations could perhaps be confined to thiol peroxidase isoforms in the cytosol and nucleus. However, it is clear specificity does exist in terms of response to a specific ROS because the loss of all 8 thiol peroxidase isoforms in yeast did not impede transcriptional responses to other distinct cellular redox changes [194]. The role of thiol peroxidases as signal transducers is depicted in Figure 2.

One regulatory 1-step redox relay event in plants has been described as was mentioned above. This is a GPX3-mediated oxidation of ABI2 in guard cells in response to ABA treatment [180]. ABA signaling involving ABI2 plays a role in HL-responses the bundle sheath cells of Arabidopsis leaves [45, 104]. However, no impact of a *GPX3* null mutant could be discerned in the HL responses of Arabidopsis [45]. In general, this observation on the ABI2 and GPX3 relationship remains a single example even though it has been over a decade since the original report.

Thiol peroxidases involved in redox relay could interact with regulatory proteins directly and different isoforms would have a cohort of different target proteins. However, the genetic redundancy among GPX and PRX genes suggest that this may not be likely. Alternatively, target regulatory proteins and a thiol peroxidase isoform could be part of a quaternary regulatory complex that includes one or more scaffold or adaptor proteins that would impose the necessary specificity. Furthermore, as with YAP1/ORP1/YBP1 relationship, the bringing of all components together into proximity would create the environment necessary for the thiol

peroxidase to act as a redox relay. This seems to us to be a promising line of enquiry for future research on HL signaling and could be a fruitful starting point to unpick the underlying transcription regulation.

Experimental problems potentially increase when redox relays are 2 or more steps, with a distributed transmission of oxidizing equivalents to a range of target proteins. One report has shown the transduction of a H_2O_2 signal to PHOSPHOLIPASE D*∂* (PLD*∂*) mediated by CYTOSOLIC GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE isoforms, GAPC1 and GAPC2, during ABA-initiated stomatal closure. *In vitro*, H_2O_2 promoted GAPC1/2 and PLD*∂* interaction and increased PLD*∂* activity [202]. *In vivo*, it would seem reasonable to suppose that H_2O_2 signalling could be mediated by a redox relay step to the GAPC isoforms. If this were to be the case, then this would be a clear example of a 2-step redox relay regulatory event in plants. If multiple step redox relay is prevalent in plants' responses to ROS and HL, then the research challenge becomes even more complex and should perhaps rely on methods of network inference by computational methods drawing on protein-protein interaction data, binary and time series transcriptomics data backed with metabolic and physiological data [123, 203 - 206].

In summary, the prospects for making progress on dissecting ROS-mediated HL signaling from chloroplasts to the nucleus are good. There are several increasingly powerful experimental tools available to researchers, most notable of which are *in vivo* fluorescent protein biosensors and other bio-imaging methods, increasingly sophisticated proteomics- and transcriptomics-based approaches, coupled with facile and flexible transient expression in leaves by agro-infection, which dramatically lowers the time taken to carry out a comprehensive molecular genetic dissection of signaling. This means that signaling studies around HL (and

other plant-environment interactions), ROS and chloroplasts can take its place alongside the pioneering work being done in yeast and animal cell systems. This should benefit all researchers, not just those of a green disposition.

A final word- phototoxicity in mammalian cells and what it tells us about retrograde signalling

Direct comparisons between plant and mammalian systems are necessarily fraught with concessions. At first sight, mammalian systems have no HL studies *per se*, as only small subsets of mammalian cells are subjected to light. But by casting the net wider, we can draw insight from several different fields. Much initial knowledge of how light affects mammalian cells stems from the study of dermatological phototoxicity [207]. By contrast, photodynamic therapy seeks to exploit the ample generation of a range of ROS through illumination and selective photosensitizers in order to destroy abnormal cells. Consequently, this field is also a rich source for studies of localised ROS damage to cell populations, tissues and organs [6, 208]. A third field has emerged with the wide-spread use of fluorescence microscopy, which relies on fluorescent proteins or dyes which in their excited state produce ROS, damaging chemical structure and biological function of proximal biomolecules. This type of phototoxicity (or more precisely fluorotoxicity) has important methodological consequences, as it is a serious constraint in live imaging, and low-light microscopy techniques are often less damaging than those using HL intensities [209].

Comparing studies in these diverse fields can be used to establish the similarities and discrepancies between different kingdoms to cope with, as well as exploit, ROS. This may reveal broadly applicable strategies, but is also certain to be complicated by significantly different responses and tolerances to illumination not only within the same kingdom, but even within the same family, species or cell line. For example,

the human HeLa cell line is 5-7 times more resistant to photodamage than COS-7 and U2OS cells [210]. Even within the same cell line, subcellular localization of the fluorophore can significantly change photodamage threshold levels, as $^1O_2$ close to lipids has a lifetime about twice that in aqueous conditions. Therefore, at the cellular level many of the interactions with light, both animal and plant cells may have more commonality than at first sight becomes apparent. Figure 3 illustrates this. Thus, carrying out a comparison of the responses to irradiation of the photosynthetic and non-photosynthetic eukaryotes can be a fruitful line of study united by the opportunities and limitations imposed by ROS produced in response to irradiation.

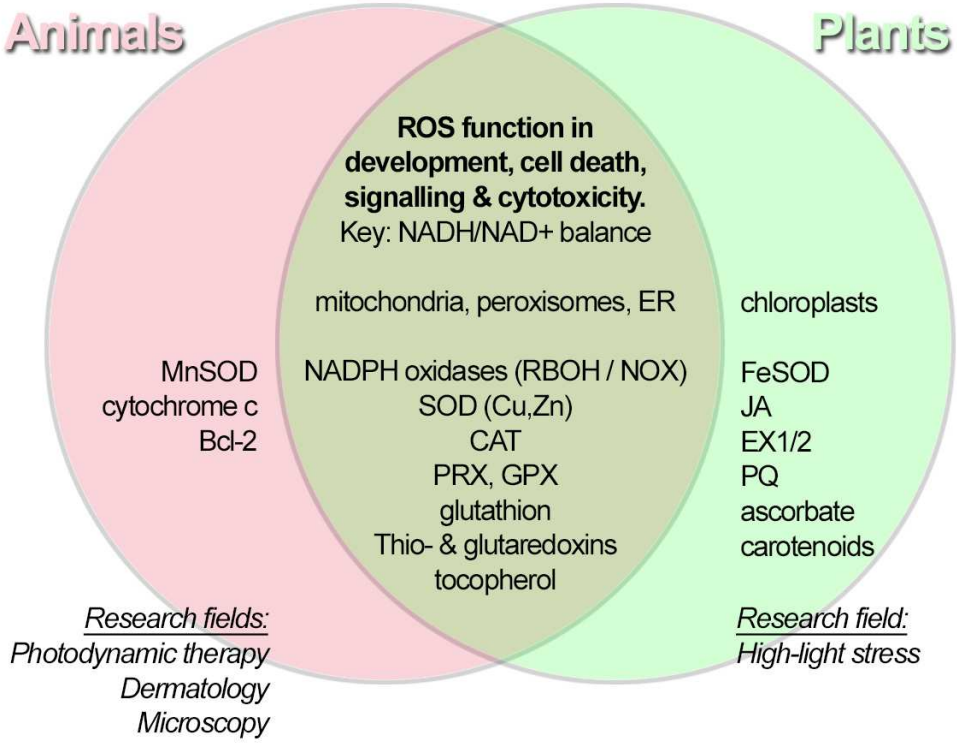


Fig. 3 A (non-exhaustive) overview of shared and different characteristics between plant and animal ROS regulation mechanisms, organelles and research fields.

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