

# Chloroplast ROS and stress signaling

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## ABSTRACT

Chloroplasts overproduce reactive oxygen species (ROS) under unfavorable environmental conditions, and these ROS are implicated in both signaling and oxidative damage. There is mounting evidence for their roles in translating environmental fluctuations into distinct physiological responses, but their targets, signaling cascades, and mutualism and antagonism with other stress signaling cascades and within ROS signaling remain poorly understood. Great efforts made in recent years have shed new light on chloroplast ROS-directed plant stress responses, from ROS perception to plant responses, in conditional mutants of *Arabidopsis thaliana* or under various stress conditions. Some articles have also reported the mechanisms underlying the complexity of ROS signaling pathways, with an emphasis on spatiotemporal regulation. ROS and oxidative modification of affected target proteins appear to induce retrograde signaling pathways to maintain chloroplast protein quality control and signaling at a whole-cell level using stress hormones. This review focuses on these seemingly interconnected chloroplast-to-nucleus retrograde signaling pathways initiated by ROS and ROS-modified target molecules. We also discuss future directions in chloroplast stress research to pave the way for discovering new signaling molecules and identifying intersectional signaling components that interact in multiple chloroplast signaling pathways.

**Keywords:** ROS,  $^1\text{O}_2$ ,  $\text{H}_2\text{O}_2$ , oxidation, operational retrograde signaling, proteostasis

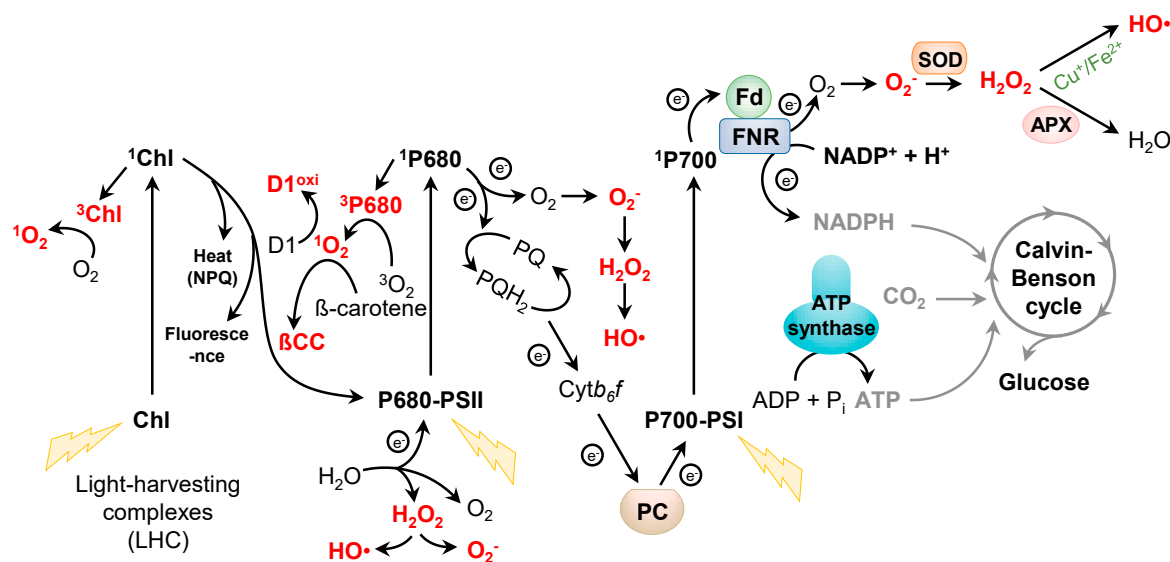
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## INTRODUCTION

Oxygenic photosynthesis provides oxygen and food for most life forms through the use of sunlight,  $\text{CO}_2$ , and water. Although oxygenic photosynthesis is a life-supporting process, inefficient utilization of sunlight yields harmful by-products, namely reactive oxygen species (ROS). Because only a minor portion of the absorbed photons (particles representing light quanta) are used to produce chemical energy in chloroplasts, excess photons must be safely dissipated (Apel and Hirt, 2004; Asada, 2006; Triantaphylides and Havaux, 2009; Kim and Apel, 2013; Khorobrykh et al., 2020). A combination of spontaneous and directed dissipation processes scatters the excess sunlight to some extent, but not entirely, inevitably producing ROS, especially under adverse environmental conditions. These excess ROS tend to oxidize macromolecules in their vicinity, thereby significantly damaging the structural and functional integrity of the chloroplast. In addition to being implicated in chloroplast damage, ROS are also involved in signaling that alerts other subcellular compartments to readjust whole-cell metabolic homeostasis and repair or turn over damaged macromolecules. Photorespiration and oxidative phosphorylation also lead to ROS production in peroxisomes and mitochondria, respectively. Thus, understanding ROS compartmentalization, crosstalk, and organelle-specific effects is crucial for understanding ROS-triggered plant stress responses.

During plastid acquisition by early eukaryotes, the plastid genome underwent significant reduction by endosymbiotic gene transfer to the host genome and by simple loss compared to its cyanobacterial ancestor (Timmis et al., 2004). Less than 5% of the original genes remain in the plastid genome, encoding mostly housekeeping and photosynthesis-associated proteins. As a result, the assembly of protein complexes in plastids necessitates, when required, the coordinated expression of nuclear and plastid genomes, referred to as genome-coupled expression (Chan et al., 2016a). This coordination is crucial for the punctual assembly of photosynthetic complexes during chloroplast biogenesis (e.g., during de-etiolation of dark-grown etiolated seedlings), which is critical for sustaining a photoautotrophic lifestyle. Likewise, dysfunctional plastids with attenuated transcription and translation require the equivalent repression of nuclear genes that encode cognate companions (Oelmüller and Mohr, 1986; Susek et al., 1993). All these related findings indicate that the functional status of the plastid is transmitted to the nucleus for genome-coupled expression, a process referred to as retrograde signaling. This process is further divided into biogenic and operational retrograde signaling (BRS and ORS) (Pogson et al.,

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**Figure 1. ROS metabolism in the chloroplast.**

The light energy captured in the photosystem II (PSII) light-harvesting antenna complex (LHC) drives photosynthetic electron transfer. Upon absorption of light energy, the chlorophyll (Chl) molecule converts to the singlet-state Chl ( $^1\text{Chl}$ ). Part of the excess energy is re-emitted as fluorescence or dissipated as heat, a process referred to as non-photochemical quenching (NPQ). Alternatively, the spontaneous decay of  $^1\text{Chl}$  results in triplet excited state  $^3\text{Chl}$  with an increased lifespan (Muller et al., 2001).  $^3\text{Chl}$  is quenched by carotenoids in the LHC, returning to its ground state. Otherwise,  $^3\text{Chl}$  transfers the absorbed energy to the ground state of  $\text{O}_2$ , producing  $^1\text{O}_2$ . The LHC-generated  $^1\text{O}_2$  can also be quenched by carotenoids. Energy that reaches the PSII RC Chl (P680) from the antenna complex also leads to the formation of  $^1\text{O}_2$  via  $^3\text{P680}$  when the electron ( $e^-$ ) transfer chain becomes over-reduced.  $^1\text{O}_2$  then oxidizes  $\beta$ -carotene and D1 proteins in its vicinity.  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  can be generated through two-electron oxidations of  $\text{H}_2\text{O}$  at the electron donor side of PSII and a one-electron reduction of  $\text{O}_2$  at the acceptor side of PSII, respectively. The subsequent conversion of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  into other forms of ROS is described in Pospisil (2009). PSI is also involved in the production and detoxification process of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ . The photosystem I (PSI) electron acceptor ferredoxin (Fd)-NADP $^+$  oxidoreductase (FNR) transfers electrons from ferredoxin to NADP $^+$  to produce NADPH. NADPH and ATP produced by ATP synthase are used to produce glucose as the final product of photosynthesis via the Calvin-Benson cycle. To maintain PSII in a partly oxidized form under conditions in which the electron transfer chain becomes over-reduced, FNR transfers an electron to  $\text{O}_2$ , thereby generating  $\text{O}_2^-$ .  $\text{O}_2^-$  is further converted into  $\text{H}_2\text{O}_2$  by SUPEROXIDE DISMUTASES (SODs). ASCORBATE PEROXIDASES (APXs) then contribute to  $\text{H}_2\text{O}_2$  scavenging through the ascorbate-glutathione cycle (Apel and Hirt, 2004).  $\text{OH}^\bullet$  is generated via the Fenton reaction by the interaction of  $\text{H}_2\text{O}_2$  with reduced transition metal ions such as  $\text{Fe}^{2+}$  and  $\text{Cu}^+$ .  $\beta\text{CC}$ ,  $\beta$ -cyclocitral;  $\text{PQH}_2$ , plastoquinone; PQ, oxidized form of plastoquinone;  $\text{Cyt}b_6f$ , cytochrome  $b_6f$  complex; PC, plastocyanin.

2008; Kleine and Leister, 2016). Whereas BRS is critical for assembling chloroplast complexes during development, ORS is generally associated with stress and mainly directed by mature, photosynthesizing chloroplasts. The discovery of stress-associated ORS pathways has established chloroplasts as environmental sensors, translating perceived environmental stimuli as distinct forms of signals. Therefore, chloroplast signaling may make a significant contribution to cellular homeostasis and plant resilience upon exposure to stress. In addition to ROS, chloroplasts also produce other stress-related signaling molecules such as reactive carbonyl species, reactive nitrogen species (RNS), reactive sulfur species (RSS), volatile compounds, secondary metabolites, and precursors of stress hormones such as salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA). Shedding further light on how and when these signaling-associated molecules are synthesized under distinct stress conditions may provide essential clues to understanding the impact of chloroplast stress signaling on the broad spectrum of plant stress responses.

## CHLOROPLAST ROS: PRODUCTION AND OXIDATIVE DAMAGE

Chloroplasts produce ROS through both photosystems because of the excess photons trapped in photosystem (PSII) and the

electron sink to molecular oxygen via photosystem I (PSI) (Apel and Hirt, 2004; Asada, 2006; Triantaphylides and Havaux, 2009). Excess energy in PSII leads to the formation of the triplet state of light-excited chlorophyll ( $^3\text{Chl}$ ) in the PSII light-harvesting antenna complex (LHC) and of P680 Chl molecules ( $^3\text{P680}$ ) in the reaction center (PSII RC) (Figure 1). Because of their increased lifespan, these  $^3\text{Chl}$  molecules can transfer the absorbed energy to ground-state oxygen ( $\text{O}_2$ ), facilitating the production of highly reactive singlet oxygen ( $^1\text{O}_2$ ) (Figure 1). A group of carotenoids closely associated with the antenna complex can quench  $^3\text{Chl}$  and the  $^3\text{Chl}$ -produced  $^1\text{O}_2$ , whereas PSII RC-generated  $^1\text{O}_2$  oxidizes  $\beta$ -carotene and D1 proteins (Dogra et al., 2018) (Figure 1). In PSII,  $^1\text{O}_2$  formation by energy transfer is well described, and the production of superoxide anion ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $\text{OH}^\bullet$ ) by PSII has also been demonstrated (Pospisil et al., 2004; Pospisil, 2009, 2014, 2016). Electron leakage on the PSII electron acceptor side produces  $\text{O}_2^-$ , which undergoes dismutation to  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  is then further converted to  $\text{OH}^\bullet$  by non-heme iron. On the PSII electron donor side, incomplete water oxidation results in the formation of  $\text{H}_2\text{O}_2$ , which is further reduced to  $\text{OH}^\bullet$  (Pospisil, 2016). The molecular basis for ROS production and the impact of ROS on target molecules in PSII provide new insight into the protection and repair of PSII. Interestingly, the phosphorylation and subsequent

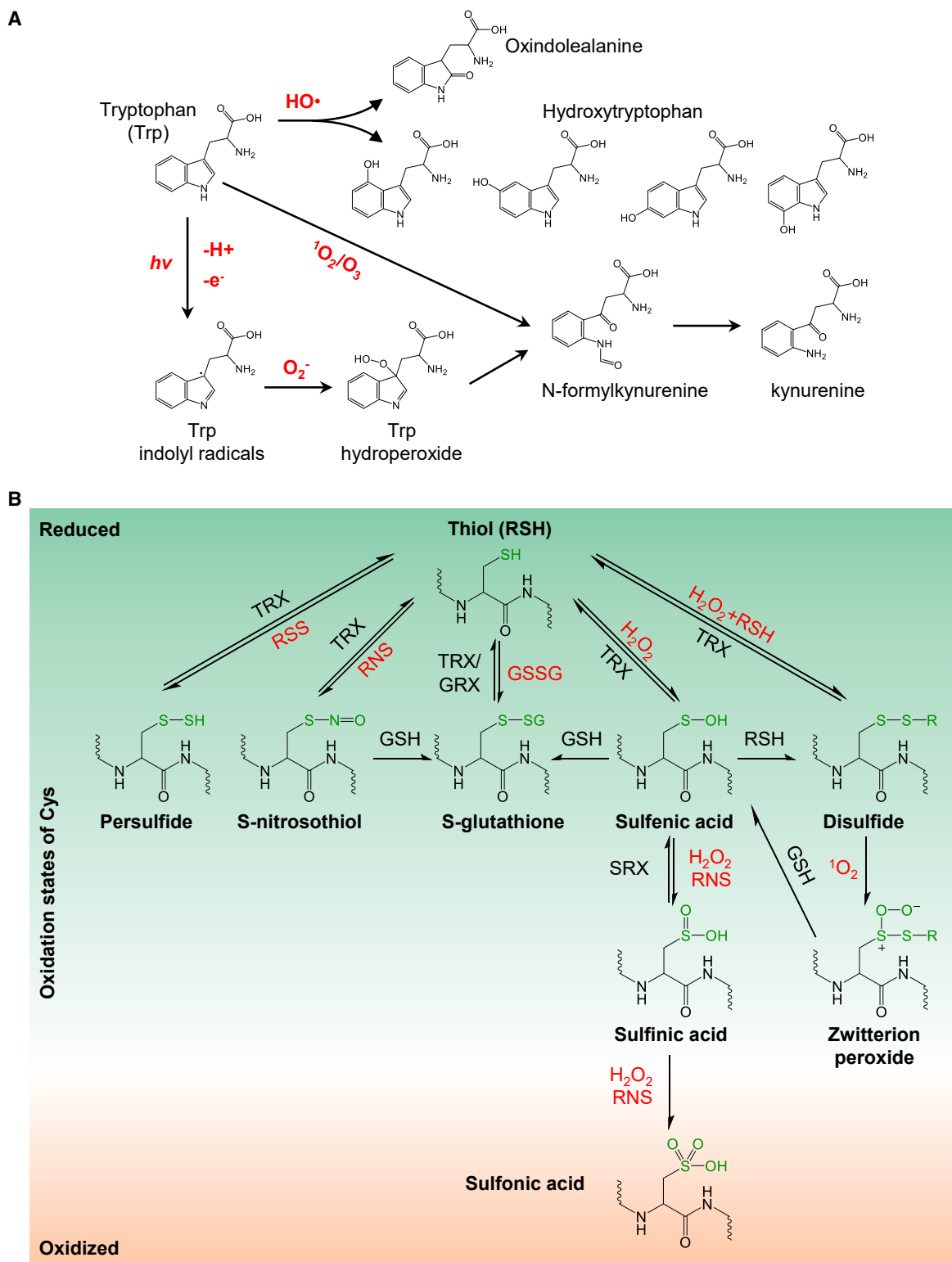
conformational changes of the D1 protein during PSII repair were found to be critical for inhibiting  $O_2^-$  production at the PSII acceptor side (Chen et al., 2012). Consistent with this finding, a rice STN8 kinase mutant defective in D1 phosphorylation exhibits elevated  $O_2^-$  levels upon exposure to high-light stress (Poudyal et al., 2016).

In PSI, the stepwise production of  $O_2^-$  and  $H_2O_2$  depends on ineffective photochemical and non-photochemical quenching (NPQ) (Havaux et al., 1991), giving away PSII-derived electrons to  $O_2$  through PSI-associated electron transport components (Figure 1). While SUPEROXIDE DISMUTASEs (SODs) catalyze the dismutation of  $O_2^-$  to  $O_2$  and  $H_2O_2$ , thylakoid membrane-associated and stromal ASCORBATE PEROXIDASEs (APXs) function in  $H_2O_2$  detoxification through the ascorbate–glutathione cycle (Ape and Hirt, 2004) (Figure 1). Such an electron sink (also called the water–water cycle) is critical to balancing oxidation and reduction of the electron transport chain to maximize photosynthetic efficiency (Asada, 2006). The highly abundant stromal 2-Cys peroxiredoxins are also known to detoxify  $H_2O_2$  in the presence of thioredoxin and NADPH-dependent thioredoxin reductase (NTRC) (Dietz et al., 2002; König et al., 2002; Broin and Rey, 2003). Chloroplasts that lack the cognate thioredoxin or NTRC thus experience overoxidation of 2-Cys peroxiredoxins, leading to increased lipid peroxidation and oxidative damage to the thylakoid membrane. Like 2-Cys peroxiredoxins, glutathione peroxidases are thought to catalyze  $H_2O_2$  reduction and to reduce oxidative damage (Chang et al., 2009). Inefficient photochemical quenching under various stress conditions results in the over-reduction of the electron transport chain. The excess energy trapped in PSII then promotes  $^1O_2$  generation, thus also promoting the oxidation of PSII-associated electron acceptors.  $OH^\bullet$ , the most reactive free radical, is produced from  $H_2O_2$  through the Fenton reaction in the presence of reduced transition metal ions such as copper ( $Cu^+$ ) or iron ( $Fe^{2+}$ ) that are generated by the  $O_2^-$ -dependent reduction of  $Cu^{2+}$  and  $Fe^{3+}$  (Figure 1). Although chloroplasts evolved with antioxidants and scavenging systems to maintain ROS homeostasis, severe or long-lasting stress conditions can rapidly deplete detoxifiers and inactivate scavenging systems. Hence, adversity results in ROS overproduction and enhanced chloroplast damage because of the reactivity of ROS toward macromolecules in their vicinity.

Among these chemically distinct ROS,  $^1O_2$  is primarily responsible for photodamage to proteins and lipids in chloroplasts (Triantaphylides and Havaux, 2009). Unlike  $H_2O_2$ ,  $^1O_2$  has an extremely short lifespan and diffusion distance, limiting its release into the cytosol. Given  $^1O_2$  production in PSII and its immense reactivity toward electron-rich molecules, the rapid turnover rate of the D1 protein in PSII RC, regardless of light intensity, is attributed to the constant generation of  $^1O_2$  as a by-product of photosynthesis (Dreaden et al., 2011; Kale et al., 2017; Kumar et al., 2021; Mitra et al., 2021). Not only D1 but also other PSII core proteins (such as D2, CP43, and CP47), polyunsaturated fatty acids (PUFAs), and other pigments (including  $\beta$ -carotene) can be oxidized by excess  $^1O_2$  (Lupinkova and Komenda, 2004; Duan et al., 2019).  $^1O_2$  oxidizes PUFAs, such as linoleic acid and linolenic acid, thereby producing reactive electrophile species (RES) that are implicated in cellular signaling networks (Dogra et al., 2018).

Specific RES molecules appear to induce detoxification responses, conferring adaptive stress response to photo-oxidative stress. Similar to carotenoids, antioxidants such as  $\alpha$ -tocopherol, ascorbate, and glutathione are known to be oxidized by  $^1O_2$ , generating their own oxidized derivatives. More information regarding the  $^1O_2$ -dependent oxidation of biomolecules is presented in Dogra et al. (2018). When the photon utilization and detoxification system becomes impaired under various stress conditions, oxidation of PSII core proteins may compromise the entire photosynthetic process unless the photosystem is reassembled rapidly by PSII quality control (also known as the PSII repair cycle) (Kato and Sakamoto, 2018). To date, research on PSII quality control has focused primarily on phosphorylation and dephosphorylation of the photodamaged D1 protein, direction of damaged PSII core migration from the grana core (appressed regions of the grana) to the grana margin (non-appressed regions), and turnover by the membrane-bound heterohexameric FtsH protease (Li et al., 2018). However, it remains unknown how ROS-driven oxidation of the D1 protein initiates PSII quality control. Perhaps its oxidation drives a conformational change of the PSII core, leading to the activation of PSII quality control.

Upon generation,  $^1O_2$  oxidizes only specific amino acid residues. Aromatic and hydrophobic amino acid residues (including tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe)), histidine (His), methionine (Met), and cysteine (Cys) are known to be oxidized by  $^1O_2$  (Michaeli and Feitelson, 1994). Among these  $^1O_2$ -sensitive residues, Trp is reported to scavenge  $^1O_2$  by both physical quenching and chemical reactions, rendering the Trp residue highly susceptible and rapidly reactive with  $^1O_2$ . Given the short lifespan of  $^1O_2$  (~200 ns) (Li et al., 2012; Telfer, 2014), a relatively small physical distance between Trp and  $^1O_2$  is a precondition for oxidation of the Trp residue *in vivo*. In addition, the Trp residue must be exposed on the surface of the target protein. Nonetheless,  $^1O_2$ -driven oxidation of Trp leads to cleavage of the indole ring, generating oxidized Trp variants such as the dihydro-hydroxy derivative *N*-formylkynurenine (NFK) and kynurenine, with corresponding mass shifts of +32 and +4 Da, respectively (Perdivara et al., 2010; Dreaden et al., 2011; Dogra et al., 2019) (Figure 2A). Because Trp residues have been implicated in various biological processes, including protein–protein interaction and anchorage of peripheral membrane proteins on the lipid bilayer (Samanta and Chakrabarti, 2001; Feng et al., 2002; Clark et al., 2003; Dogra et al., 2019), cleavage of the indole ring could modulate Trp-linked biological processes. However, as mentioned above, the site of  $^1O_2$  generation and the physical distance to the Trp residue of the target protein must first be considered before further investigation. Similar to  $^1O_2$ , ozone ( $O_3$ ) also oxidizes Trp residues, generating NFK and kynurenine (Figure 2A), and ultraviolet light (UV) oxidizes the indole side chain of Trp. Direct UV absorption produces a triplet state of Trp ( $^3Trp$ ), which rapidly undergoes electron transfer reactions and deprotonation to generate neutral Trp indolyl radicals (Figure 2A). The superoxide radical anion ( $O_2^-$ ) further reacts with Trp indolyl radicals, forming Trp hydroperoxide and its subsequent end products NFK and kynurenine (Figure 2A). The interaction between  $HO^\bullet$  and Trp produces distinct oxidized Trp variants, such as oxindolealanine or several hydroxytryptophan molecules (Berlett et al., 1996; Ehrenshaft





et al., 2015) (Figure 2A). The complex flow of Trp oxidation pathways suggests that PSI-associated proteins may also undergo  $O_2^{\cdot-}$ -dependent oxidation. In addition to Trp residues, a recent study demonstrated that another aromatic amino acid residue, tyrosine (Y) 246 in the D1 protein, can also be oxidized by  $OH^\bullet$  and  $O_2^{\cdot-}$  in PSII (Kumar et al., 2021). Its oxidation in the *Arabidopsis vte1* mutant suggested the significance of  $\alpha$ -tocopherol for preventing oxidative modification of the D1-Y246 residue. Considering the generation of other ROS in PSII through different mechanisms (Kumar et al., 2021), the oxidative modifications of PSII proteins under various stress conditions remain to be further explored.

The diffusible and long-lived  $H_2O_2$  produced through the electron sink in PSI primarily oxidizes the thiol group of a Cys residue (Wang et al., 2012). However, given its relatively long lifespan,  $H_2O_2$  may oxidize chloroplast proteins carrying Cys residues by chance. It is important to note that not only ROS but also other oxidizing agents such as RNS, RSS, and an oxidant—oxidized glutathione—can oxidize the thiol group of Cys, as shown in Figure 2B. Nonetheless,  $H_2O_2$  induces reversible and irreversible oxidations of Cys residues (Figure 2B). Whereas irreversible Cys oxidation would facilitate protein degradation, reversible Cys oxidation may involve the on/off switching of biological processes or redox-sensitive signaling (Pajares et al., 2015). Bacterial elongation factor G (EF-G, including that of cyanobacteria) has been shown to form an ROS-dependent and thioredoxin (Trx)-reversible interdisulfide bond between two Cys residues (Nishiyama et al., 2006; Kojima et al., 2009; Nagano et al., 2012). The disulfide bond (DSB) inactivates EF-G, thereby slowing protein translation. The Trx-dependent reactivation of EF-G enables the versatile regulation of translation in response to ever-changing environmental conditions. Interestingly,  $^1O_2$  can oxidize a DSB, generating reactive zwitterion peroxide ( $R-S^+-OO^-$ ) (Figure 2B). This oxidized protein can form a disulfide crosslink with thiol-containing proteins (Jiang et al., 2021). This process can be reversed by reduced glutathione (GSH) (Figure 2B). Consecutive thiol oxidations by different ROS may escalate chloroplast damage by increasing the oxidation of target proteins, further impairing proteostasis. However, it remains unclear whether zwitterion peroxide occurs in plants.

## OPERATIONAL RETROGRADE SIGNALING PATHWAYS

### $\beta$ CC and MEcPP pathways

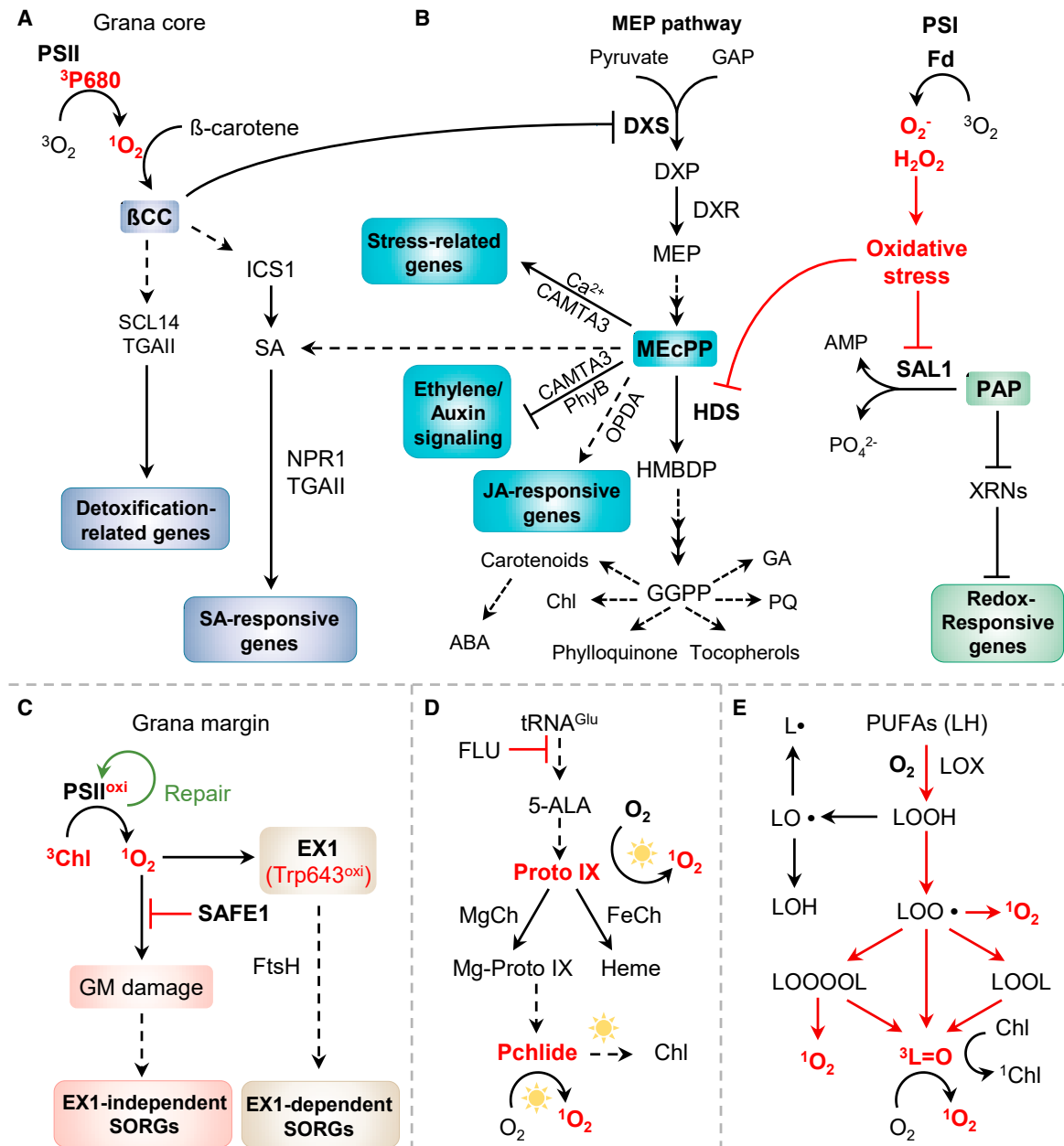
The discovery of two *Arabidopsis* mutants, *chlorina1* (*chl1*) and *fluorescent* (*flu*), changed the classical view of  $^1O_2$  from toxin to signal and led us to gain insights into two distinct  $^1O_2$ -signaling pathways (Meskauskiene et al., 2001; Ramel et al., 2013b). The *chl1* mutant genome contains a mutation inactivating Chl *a* oxygenase, the enzyme that catalyzes Chl *b* synthesis. Because Chl *b* is required for the assembly of the peripheral

light-harvesting antenna complexes, the *chl1* mutant exhibits exacerbated photoinhibition in PSII RC under light-stress conditions (Ramel et al., 2013a, 2013b). Whereas excess light is required to generate  $^1O_2$  in PSII RC of *chl1*, the *flu* mutant produces  $^1O_2$  conditionally in the thylakoid membrane upon a dark-to-light shift (Dogra et al., 2018). FLU protein blocks the  $Mg^{2+}$  branch of tetrapyrrole synthesis in the dark, and the *flu* mutant therefore accumulates free protochlorophyllide (Pchlde) in the dark because the next conversion step from Pchlde to chlorophyllide requires light-dependent Pchlde oxidoreductase (POR) enzymes (Meskauskiene et al., 2001; Goslings et al., 2004). The excess free Pchlde serves as a photosensitizer, transferring absorbed light energy to molecular oxygen and thereby generating highly reactive  $^1O_2$ . Through an unbiased forward genetic screen,  $^1O_2$ -triggered stress responses, such as nuclear gene expression changes, cell death in young seedlings, and growth inhibition in mature *flu* mutant plants, were shown to be mediated by a nuclear-encoded chloroplast EXECUTER1 (EX1) protein (Wagner et al., 2004). Collectively,  $^1O_2$ -triggered ORS pathways can be investigated under both photoinhibitory and non-photoinhibitory conditions by exploiting *chl1* and *flu*, respectively.

Excess light-driven  $^1O_2$  production leads to oxidation and cleavage of  $\beta$ -carotene in the PSII RC in the grana core, producing various carbonyl products, namely apocarotenoids, in *chl1* mutant and wild-type (WT) plants (Ramel et al., 2012a, 2012b, 2013a, 2013b) (Figure 3A). Among these products,  $\beta$ -cyclocitral ( $\beta$ CC) and dihydroactinidiolide (DhA) have been shown to be biologically active volatile compounds that mediate ORS (Ramel et al., 2012b).  $\beta$ CC- and DhA-induced nuclear transcripts confer plant tolerance under various abiotic stress conditions. The findings that PSII produces  $^1O_2$  and that  $^1O_2$  generates volatile signaling compounds through the oxidation of  $\beta$ -carotene suggest that PSII acts as a sensory apparatus, translating critical levels of  $^1O_2$  into the form of volatile signals. The excess light-released  $\beta$ CC induces detoxification-responsive genes via the GRAS protein transcription factor SCARECROW LIKE 14 (SCL14), primarily to mitigate lipid peroxidation-driven toxicity (D'Alessandro et al., 2018) (Figure 3A). The class II TGA factors TGA2, TGA5, and TGA6 recruit SCL14 to the promoters of SCL14 target genes that encode proteins required for detoxification responses (Fode et al., 2008). The SCL14-induced ANAC102 transcription factor is crucial for inducing detoxification-responsive genes (Christianson et al., 2009; D'Alessandro et al., 2020).

$\beta$ CC also rapidly induces a nuclear gene encoding ISOCHORISMATE SYNTHASE 1 (ICS1), a key enzyme involved in SA synthesis in chloroplasts. Induction of ICS1 leads to increased cellular SA levels and activation of NPR1 (NONEXPRESSER OF PR GENES1, an SA receptor), conferring plant acclimation to high-light stress (Lv et al., 2015; Mitra et al., 2021) (Figure 3A). This finding, along with the other reports mentioned above, confirms that  $\beta$ CC can activate

agent, cleaving the disulfide bond and restabilizing the conformation. Different oxidations convert the RSH into oxidized Cys variants, including persulfide, S-nitrosothiol, S-glutathione, sulfenic acid, disulfide, sulfinic acid, zwitterion peroxide, and sulfonic acid. Reversible and irreversible modifications are indicated. The persulfide, S-nitrosothiol, S-glutathione, sulfenic acid, and disulfide can be reduced by small ubiquitous redox proteins such as thioredoxin (TRX) or glutaredoxin (GRX) (Klatt and Lamas, 2000). Sulfiredoxin (SRX) is responsible for reducing sulfinic acid into sulfenic acid (Biteau et al., 2003). Sulfenic acid can be further oxidized to an irreversible form of sulfonic acid, affecting protein functionality, conformation, and turnover rate.



**Figure 3. Chloroplast ROS pathways.**

**(A)**  $\beta\text{CC}$ -mediated ORS. High-light stress leads to a burst of  $^1\text{O}_2$  in PSII enriched in the grana core, resulting in the accumulation of oxidized  $\beta$ -carotene-derived volatile products such as  $\beta\text{CC}$ .  $\beta\text{CC}$  induces detoxification genes via the GRAS protein SCL14 and TGAI1 transcription factors.  $\beta\text{CC}$  also induces the *ICS1* gene, encoding a key SA synthesis enzyme. The increased SA level then evokes SA-responsive genes through the SA receptor NPR1 and TGAI1 transcription factors.  $\beta\text{CC}$  also inhibits DXS, the rate-limiting enzyme in the MEP pathway.

**(B)** PAP- and MEcPP-mediated ORS pathways. Environmental factors, such as high light and drought, increase  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  levels in PSI, changing the chloroplast redox status. Such oxidative stress inactivates SAL1 phosphatase and HDS in the MEP pathway, resulting in PAP and MEcPP accumulation, respectively. PAP then mediates an ORS to induce plastid redox-associated genes by blocking the RNA-degrading activity of XRNs in the nucleus. Like PAP, MEcPP migrates to the nucleus to evoke the expression of stress-responsive genes in a  $\text{Ca}^{2+}$ - and CAMTA3-dependent manner. The activation of CAMAT3 by MEcPP stabilizes the red-light photoreceptor PhyB with concurrent reductions in ethylene and auxin. MEcPP also elicits SA- and JA-responsive genes to modulate plant stress responses.

**(C)** EX1-mediated ORS in the grana margin. The  $^1\text{O}_2$ -dependent oxidation of the Trp643 residue (Trp643<sup>oxi</sup>) in EX1 leads to FtsH2-dependent EX1 proteolysis, eliciting ORS. Another chloroplast protein, SAFE1, protects grana margin proteins from  $^1\text{O}_2$ . The loss of SAFE1 induces EX1-independent  $^1\text{O}_2$ -responsive genes (SORGs).

**(D)**  $^1\text{O}_2$  generation by tetrapyrrole intermediates. The tetrapyrrole biosynthesis (TPB) pathways are divided into Chl and heme branches. In the Chl branch, MAGNESIUM CHELATASE (MgCh) inserts  $\text{Mg}^{2+}$ , whereas in the heme branch, FERROCHELATASE (FeCh) inserts  $\text{Fe}^{2+}$ . Most of the free tetrapyrrole

(legend continued on next page)

both detoxification and SA signaling via SCL14-TGALLs and NPR1-TGALLs, respectively. Perhaps  $\beta$ CC-induced SA signaling antagonizes the lipid peroxidation-driven stress responses that are controlled in part by another stress hormone, JA, considering their well-established antagonism (Thaler et al., 2012). How Recent studies also indicate that the SA and JA signaling pathways synergistically aid plants in adapting to high-light stress (D'Alessandro et al., 2020). The synergistic activation of SA and JA signaling pathways has been found to confer basal thermotolerance in *Arabidopsis* (Clarke et al., 2009). Nonetheless, simulated herbivory (i.e., *Spodoptera littoralis*, oral secretion) also causes  $\beta$ CC release with a coincident upregulation of  $^1\text{O}_2$ -responsive nuclear genes, suggesting that herbivory can lead to  $^1\text{O}_2$  release in chloroplasts, that excess light is dispensable for  $\beta$ CC generation, and that  $\beta$ CC signaling functions in response to biotic stress.  $\beta$ CC then suppresses the plastidial methylerythritol phosphate (MEP) pathway by directly inhibiting DXS, a rate-limiting enzyme in the MEP pathway, and repressing the expression of *DXS* (Mitra et al., 2021) (Figure 3B). The MEP pathway produces essential organic compounds that are ubiquitous precursors for Chl, carotenoids (including  $\beta$ -carotene), tocopherols (antioxidants), phyloquinone, gibberellic acid (GA), and ABA, connecting the MEP pathway to various important biological processes (especially photosynthesis) (Figure 3B). Therefore, DXS inhibition by  $\beta$ CC will significantly decrease plant growth and carbon assimilation, in addition to its positive roles in SA signaling and detoxification responses.  $\beta$ CC induced by mechanical wounding has a similar effect on the MEP pathway.

The significance of the MEP pathway for photosynthesis suggests that, like PSII, MEP components may act in ORS upon perturbation of the pathway by environmental factors. Indeed, the inactivation of the MEP pathway by excess light leads to the accumulation of an intermediate, methylerythritol cyclodiphosphate (MEcPP), which has been identified as an ORS molecule (Walley et al., 2015; Jiang and Dehesh, 2021) (Figure 3B). Consistently, various oxidative stresses inhibit HYDROXYMETHYL-BUTENYL DIPHOSPHATE SYNTHASE (HDS), which catalyzes the conversion of MEcPP to 2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP), resulting in MEcPP accumulation and ORS induction (Jiang and Dehesh, 2021) (Figure 3B). Further study demonstrated that CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3) acts as a downstream signaling component of MEcPP to induce a suite of stress-related genes in the nucleus, including genes involved in the endoplasmic reticulum unfolded protein response (erUPR) (Benn et al., 2016) (Figure 3B). MEcPP also elicits JA-responsive genes upon an increased level of the JA precursor *cis*-(+)-12-oxo-phytodienoic acid (produced in chloroplasts via lipid peroxidation) but with nearly negligible levels of JA in the

MEcPP-overproducing mutant, *ceh1* (constitutively expressing *hydroperoxide lyase1*) (Lemos et al., 2016) (Figure 3B). The *ceh1* mutant has been shown to have elevated cellular SA content and SA-responsive genes (Bjornson et al., 2017) (Figure 3B). Perhaps SA signaling may antagonize or modulate JA signaling to adjust MEcPP-triggered cellular stress responses (Gil et al., 2005). A forward genetic study aimed at finding suppressors of *ceh1* demonstrated that MEcPP-dependent ORS stabilizes the red-light photoreceptor PHYTOCHROME B (PhyB) in a CAMTA- and  $\text{Ca}^{2+}$ -dependent manner, suggesting that MEcPP functions in photomorphogenic growth in addition to its role in stress responses (Jiang et al., 2019) (Figure 3B). MEcPP also reduces auxin and ethylene levels by transcriptionally repressing PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and PIF5, which are responsible for activating auxin and ethylene biosynthetic genes such as FLAVIN MONOOXYGENASEs and ACC SYNTHASEs, respectively (Figure 3B). These findings have established MEcPP as an upstream regulator that coordinates light and hormone signaling pathways. Not only stress but also developmental processes such as hypocotyl elongation and flowering are regulated by MEcPP in a dose-dependent manner (Wang et al., 2014, 2015). The multiple functions of MEcPP in adaptive plant stress responses through phytohormones and in plant growth through well-known molecular components such as PhyB suggest that MEcPP acts as a critical retrograde signal involved in the growth–stress tradeoff.

The facts that  $\beta$ CC inhibits the MEP pathway under various stress conditions and that  $\beta$ -carotene is one of the downstream products of the MEP pathway suggest a multifaceted interplay between  $^1\text{O}_2$ -triggered and MEcPP-dependent ORS pathways. For instance,  $^1\text{O}_2$ -driven  $\beta$ CC-mediated inhibition of the MEP pathway may attenuate the biosynthesis of  $\beta$ -carotene and photosynthesis, thereby repressing  $^1\text{O}_2$  generation (Figure 3A and 3B). Inhibition of the MEP pathway by  $\beta$ CC could also alter MEcPP-mediated plant stress responses that are coordinated by induced stress hormones, as mentioned above (Figure 3A and 3B). Thus, further investigation of the interlocking regulation of  $\beta$ CC- and MEcPP-mediated ORS pathways under controlled stress conditions or in mutants may unravel the complex modes of action of these two signaling molecules and their signaling cascades.

### EX1-mediated $^1\text{O}_2$ signaling and lipid peroxidation-dependent $^1\text{O}_2$ signaling pathways

$\beta$ CC is released from the photosynthetic PSII RC, which is enriched in the grana core, but the EX1 protein is exclusively localized in the grana margin (Wang et al., 2016), indicating a spatial separation of these two  $^1\text{O}_2$  sensors in the thylakoid membrane (Figure 3A and 3C). EX1 interacts with PSII core proteins, Chl synthesis enzymes

intermediates containing the macrocycle structure can act as photosensitizers upon exposure to light, thereby generating  $^1\text{O}_2$ . In the dark, the FLU protein negatively regulates the accumulation of Pchl<sub>ide</sub> by repressing the synthesis of 5-aminolevulinic acid (ALA), the universal precursor of TPB.

**(E)** Lipid peroxidation-dependent  $^1\text{O}_2$  signaling. LOX-mediated oxidation of PUFAs (LH) generates lipid hydroperoxide (LOOH). LOOH can be reduced to lipid alkoxyl radical ( $\text{LO}^\bullet$ ) in the presence of transition metals. Further reduction of  $\text{LO}^\bullet$  produces lipid radical ( $\text{L}^\bullet$ ) and lipid hydroxide (LOH). Under oxidation-prone conditions, LOOH is oxidized into  $\text{LOO}^\bullet$ , which spontaneously forms high-energy intermediates such as cyclic endoperoxide (LOOL) and linear tetroxide (LOOOOL). Both  $\text{LOO}^\bullet$  and LOOOOL may directly generate triplet ketones ( $^3\text{L}=\text{O}$ ) or  $^1\text{O}_2$  through disproportionation reactions based on the Russell mechanism (Girotti, 1985). The thermal cleavage of LOOL is also known as the source of  $^3\text{L}=\text{O}$ . As an excited-state carbonyl,  $^3\text{L}=\text{O}$  can transfer excitation energy to  $\text{O}_2$  and Chl to form  $^1\text{O}_2$  and  $^1\text{Chl}$ , respectively. GAP, glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GGPP, geranylgeranyl pyrophosphate; GA, gibberellic acid; tRNA<sup>Glu</sup>, glutamyl-tRNA.

(especially POR enzymes), the PSII quality control-associated FtsH protease, and a protein elongation factor in the grana margin (Wang et al., 2016; Dogra et al., 2018, 2019). Because all of these proteins are required for PSII quality control and reassembly, EX1 is thought to sense  $^1\text{O}_2$  generated by free Chl molecules or intermediate tetrapyrroles released accidentally during PSII quality control or reassembly (Dogra and Kim, 2019; Wang and Apel, 2019) (Figure 3C). Indeed, it is unknown whether the P680 Chl molecule must be replaced during D1 degradation/co-translational insertion or sequestered before recycling to avoid  $^1\text{O}_2$  production. Identifying a suite of Chl-binding proteins linked to PSII quality control suggests that such proteins may quench  $^1\text{O}_2$  in the grana margin or sequester free Chl molecules before recycling, thereby sustaining/protecting PSII repair (Yao et al., 2007, 2012; Nixon et al., 2010; Knoppova et al., 2014). In the event that stress factors disturb any steps in PSII quality control (e.g., sequestration of free tetrapyrroles by Chl-binding proteins), the accidentally released free Chl or tetrapyrrole molecules may generate  $^1\text{O}_2$  in the grana margin, enabling an EX1-dependent ORS independent of  $\beta\text{CC}$ . ROS also deactivate cyanobacterial EF-G by promoting intra-disulfide bond formation between two Cys residues (Nishiyama et al., 2006, 2011). Assuming that EF-G oxidation occurs in plant chloroplasts, the compromise of PSII reassembly by EF-G oxidation may increase the probability of free tetrapyrrole release in the grana margin, leading to a release of  $^1\text{O}_2$  in or on the grana margin and thereby stimulating EX1-dependent ORS. This notion encourages further study of chloroplast EF-G in the context of PSII repair,  $^1\text{O}_2$  generation, and EX1-dependent signaling from the grana margin.

Dogra et al. (2019) proposed a model of how EX1 protein senses  $^1\text{O}_2$  to initiate downstream physiological events, including cell death. Mass spectrometry analysis accurately determined an oxidation-prone Trp643 residue located in the  $^1\text{O}_2$  sensor domain (previously annotated as the domain of unknown function 3506) of EX1 (Figure 3C). In the *flu* mutant background, the conditional release of  $^1\text{O}_2$  in chloroplasts increases Trp643 oxidation, whereas Trp643 is maintained intact in darkness (Goslings et al., 2004; Dogra et al., 2019). The replacement of Trp643 with  $^1\text{O}_2$ -insensitive but hydrophobic residues such as alanine (Ala) and leucine (Leu) completely abrogates  $^1\text{O}_2$ -triggered stress responses in the *flu* mutant-like *flu ex1* double mutant. The resulting FtsH2-dependent EX1 degradation in response to  $^1\text{O}_2$  appears to be an integral part of  $^1\text{O}_2$  signaling (Wang et al., 2016; Dogra et al., 2017; Kim, 2019), suggesting that EX1 proteolysis may promote the release of an actual signaling molecule (Figure 3C). Despite the apparent abrogation of  $^1\text{O}_2$  signaling by either EX1(Trp643Leu) or EX1(Trp643Ala), it is unclear whether Trp643 oxidation triggers FtsH-dependent EX1 turnover. Perhaps its replacement with a less oxidation-prone Phe (Ehrenshaft et al., 2015) may provide a piece of additional information to address this question. Among  $^1\text{O}_2$ -responsive genes induced by EX1-mediated ORS, *SIGMA FACTOR BINDING PROTEIN 1* (*SIB1*), *WRKY33*, and *WRKY40* are also known to be rapidly induced by SA, suggesting possible crosstalk or interplay between  $^1\text{O}_2$  (EX1-dependent) and SA signaling. SA-induced *SIB1* accumulates in both the nucleus and the chloroplasts (Lv et al., 2019). Nuclear *SIB1* reinforces the expression of photosynthesis-associated nuclear genes (PhANGs), whereas chloroplast-localized *SIB1* represses photosynthesis-associated plastid genes (PhAPGs), leading to genomes-uncoupled expression (especially of PSII

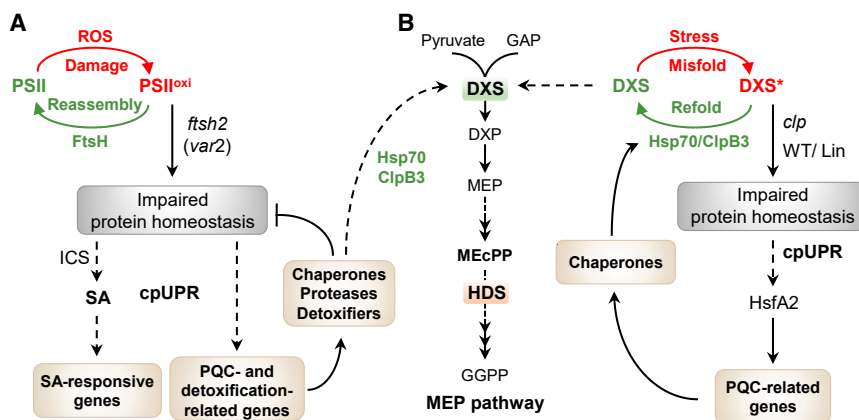
components) and  $^1\text{O}_2$  generation due to impaired PSII stoichiometry. This genomes-uncoupled expression of PhANGs and PhAPGs appears to potentiate SA-mediated stress responses by activating EX1-mediated  $^1\text{O}_2$  signaling, collectively providing a first glimpse of the impact of genomes-uncoupled expression. In agreement with this finding, *Arabidopsis* mutants deficient in PSII proteostasis exhibit reinforced SA signaling in a light-dependent manner (Duan et al., 2019) (see “Chloroplast protein homeostasis and the unfolded protein response”).

A recent forward genetic study aimed at finding EX1-independent  $^1\text{O}_2$  signaling unveiled a novel chloroplast protein, dubbed SAFEGUARD 1 (SAFE1), which protects grana margin-associated proteins from oxidative damage and degradation (Wang et al., 2020) (Figure 3C). Interestingly, together with other proteins in the grana margin, the light-dependent POR enzymes that catalyze Pchlde conversion to chlorophyllide undergo rapid proteolysis upon release of  $^1\text{O}_2$  in the *flu ex1 safe1* triple mutant. Consistently, the loss of SAFE1 re-evokes  $^1\text{O}_2$  signaling in the *flu ex1* double mutant background, perhaps through increased oxidative damage to other proteins or lipids in the grana margin. Indeed, upon release of  $^1\text{O}_2$ , an increased number of plastoglobules (oxidative stress markers) were observed in *flu ex1 safe1* compared with *flu* and *flu ex1*. However, given its exclusive localization in the stroma and the absence of recognizable functional domains in SAFE1, the mechanism underlying the reactivation of  $^1\text{O}_2$  signaling in *flu ex1 safe1* requires further exploration.

In *flu*, a  $^1\text{O}_2$  burst upon a dark-to-light shift results in chloroplast leakage, manifested by the release of stromal proteins to the cytosol, followed by vacuole rupture (Kim et al., 2012). This  $^1\text{O}_2$ -driven chloroplast leakage was abrogated by the loss of EX1, indicating that it was ORS dependent. A similar cellular process was also observed in the *ferrochelatase 2* (*fc2*) mutant owing to accumulated free protoporphyrin IX (Proto IX), as FC2 catalyzes the conversion of Proto IX to heme (Scharfenberg et al., 2015; Woodson et al., 2015) (Figure 3D). As free Pchlde generates  $^1\text{O}_2$  upon exposure to light, free Proto IX also acts as a photosensitizer. Like the *flu* mutant, the *fc2* mutant exhibits chloroplast leakage and vacuole rupture upon release of  $^1\text{O}_2$  via accumulated free Proto IX. However, despite the similar cellular process associated with cell death, EX1 was found to be dispensable for the cell death response in the *fc2* mutant (Woodson et al., 2015), indicating the presence of additional  $^1\text{O}_2$  signaling. Given the dual localization of FC2 in the envelope and thylakoid membranes (Roper and Smith, 1997), this  $^1\text{O}_2$ -triggered but EX1-independent cell death suggests that  $^1\text{O}_2$  produced in the envelope membrane rather than in the thylakoid membrane may trigger EX1-independent cell death. Therefore, further exploration of the precise location of FC2 and of the spatial accumulation of free tetrapyrroles in chloroplasts may reveal an additional  $^1\text{O}_2$  signaling pathway in addition to the  $\beta\text{CC}$ - and EX1-mediated  $^1\text{O}_2$  signaling pathways. Like EX1-independent cell death in the *fc2* mutant, OXIDATIVE SIGNAL-INDUCIBLE 1 (*OXI1*) kinase was found to mediate high-light-induced cell death in the *chl1* mutant independently of the EX1 protein (Shumbe et al., 2016).

Similar to photosynthetic  $^1\text{O}_2$ , non-photosynthetic  $^1\text{O}_2$  triggers programmed cell death accompanied by vacuole rupture (Chen and Fluhr, 2018). Multiple lines of evidence point to the generation of non-photosynthetic  $^1\text{O}_2$  in plants. It has been





**Figure 4. Impaired chloroplast proteostasis triggers an unfolded protein response.**

**(A)** ROS-driven PSII damage ( $\text{PSII}^{\text{oxi}}$ ) is inevitable during photosynthesis. The thylakoid membrane FtsH protease, a heterohexameric complex, degrades damaged PSII core proteins, promoting PSII reassembly. The *Arabidopsis var2* mutant, which lacks the major subunit of the FtsH hexamer FtsH2, accumulates oxidized PSII core proteins with a concurrent expression of genes required for chloroplast protein quality control (PQC) and ROS detoxification, a signaling pathway dubbed the chloroplast UPR (cpUPR). The *var2*-evoked cpUPR facilitates the removal of damaged chloroplast proteins, thereby restoring chloroplast proteostasis to a certain extent. Impaired PSII proteostasis causes accumulation of SA via the chloroplast isochlorismate (ICS) pathway, resulting in the enhanced expression of SA-responsive genes.

**(B)** A similar molecular response has also been shown in *Arabidopsis* and *Chlamydomonas* mutants with reduced stromal Clp protease activity. The cpUPR restores the aggregated enzyme  $\text{DXS}^*$  in the MEP pathway in both the *Arabidopsis clp* mutant and WT plants treated with lincomycin (Lin, a prokaryotic translation inhibitor). Treating WT seedlings with Lin impairs the expression of plastid-encoded Clp protease subunits, thereby repressing Clp protease activity and inducing the cpUPR. The cpUPR-induced HEAT STRESS TRANSCRIPTION FACTOR A-2 (HsfA2) positively regulates the expression of PQC-related nuclear genes. Among them, chaperones Hsp70 and ClpB3 refold the aggregated DXS enzyme. Increased levels of both chaperones are also observed in the *var2* mutant, suggesting that *var2*-driven oxidative stress may lead to aggregation of the DXS enzyme in the MEP pathway.

proposed that lipid peroxidation and its peroxy radical products cause  $^1\text{O}_2$  generation (Kanofsky and Axelrod, 1986; Miyamoto et al., 2003; Prasad et al., 2017). Consistently, wounding and osmotic stress in dark-adapted leaves and roots lead to the production of  $^1\text{O}_2$  (Flors et al., 2006; Chen and Fluhr, 2018). LIPOXYGENASE (LOX)-mediated oxidation of PUFAs also elicits electronically excited triplet carbonyls, which generate  $^1\text{O}_2$  following interaction with ground-state oxygen (Kanofsky and Axelrod, 1986; Miyamoto et al., 2003; Prasad et al., 2017) (Figure 3E). Perhaps LOX-dependent  $^1\text{O}_2$  generation occurs in dark-adapted leaves in response to wounding or in roots upon osmotic stress (Flors et al., 2006; Chen and Fluhr, 2018), where stress-related phytohormones induce the expression of LOXs (Zhou et al., 2014; Upadhyay and Mattoo, 2018). However,  $^1\text{O}_2$  formation from triplet excited carbonyls in PSII has also been demonstrated (Pathak et al., 2017; Pospisil and Yamamoto, 2017), linking lipid peroxidation-dependent  $^1\text{O}_2$  generation to photosynthesis. ROS-dependent lipid peroxidation may also induce the expression of LOXs, further reinforcing LOX-dependent  $^1\text{O}_2$  generation. Indeed, an earlier study showed rapid upregulation of *LOX2* and *LOX3* in the *flu* mutant upon release of  $^1\text{O}_2$  in chloroplasts (Danon et al., 2005). Nevertheless, given the vital role of LOXs in JA synthesis, additional crosstalk between  $^1\text{O}_2$  and JA signaling can be proposed, in addition to the crosstalk between  $^1\text{O}_2$  and SA signaling. Because  $^1\text{O}_2$  positively contributes to SA signaling, as mentioned above, either antagonism or synergism may be expected to occur between  $^1\text{O}_2$  and JA signaling.

### SAL1-dependent ORS pathway

The electron sink via PSI-associated ferredoxin generates  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , which undergo stepwise detoxification in which SODs and APXs play critical roles (Figure 1). This process is essential for maintaining PSII-associated electron acceptors in a partially oxidized state, allowing continuous electron transfer

from PSII to PSI to produce chemical energy in the form of NADPH (Figure 1). However, unfavorable growth conditions lead to the accumulation of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  in chloroplasts, perhaps because of their limited detoxification capacity. On the other hand, detoxification limitation and the existence of ORS pathways led us to consider chloroplasts as environmental sensors, translating ROS levels that exceed their detoxification capacity into signaling. The *Arabidopsis sal1* mutant, which lacks the chloroplast nucleotide phosphatase SAL1, constitutively expresses APX2 under normal growth conditions and overexpresses APX2 upon exposure to high-light stress (Estavillo et al., 2011; Chan et al., 2016a, 2016b). The lack of SAL1 results in chloroplast accumulation of one of the SAL1 substrates, 3'(2')-phosphoadenosine-5'-phosphate (PAP) (Figure 3B). This PAP inhibits nuclear 5'-to-3' EXORIBONUCLEASEs (XRNs), thereby affecting RNA catabolism and eliciting a post-transcriptionally altered nuclear transcriptome (Estavillo et al., 2011) (Figure 3B). Chan et al. (2016b) demonstrated that SAL1 undergoes ROS-dependent inactivation by forming intra- and inter-disulfide bridges at oxidation-prone Cys residues. In particular, PSI-sourced ROS notably diminish SAL1 activity in *Arabidopsis* (Figure 3B). Because high-light stress leads to the accumulation of MEcPP, PAP, and  $\beta\text{CC}$  via PSII/PSI-driven ROS, their crosstalk seems to be far more complicated than we can imagine (Figure 3A and 3B). The relative concentrations of each ROS, their lifespans, and their crosstalk with stress hormones must also be considered. Thus, mutants that constitutively overproduce these ORS molecules must have pleiotropic phenotypes rather than specific phenotypes. Consistent with this concern, recent studies on PAP-XRN ORS revealed its impact on various biological processes, including the circadian clock, iron homeostasis, ABA signaling, and drought stress responses (Pornsirirong et al., 2017; Lithauer et al., 2018; You et al., 2019; Balparda et al., 2020).

A study showed that PAP signaling contributes to ABA-mediated stress responses by upregulating multiple ABA and  $\text{Ca}^{2+}$  signaling components such as CALCIUM DEPENDENT PROTEIN KINASES (CDPKs), CALCINEURIN B-LIKE PROTEINS (CBLs), and CBL-INTERACTING PROTEIN KINASES (Pornsiriwong et al., 2017). The *sal1* mutant thus shows increased stomatal closure under excess light as well as enhanced drought resistance, implying a positive correlation between PAP and ABA signaling (Rossel et al., 2006; Pornsiriwong et al., 2017). Consistent with this notion, the inactivation of SAL1 restores the ABA responses of *Arabidopsis* mutants defective in ABA response.

As the interplay between chloroplast  $\text{H}_2\text{O}_2$  and ABA response in guard cells has been demonstrated, PAP-mediated ORS appears to activate ABA signaling upon an increase in  $\text{H}_2\text{O}_2$  in chloroplasts.

## CHLOROPLAST PROTEIN HOMEOSTASIS AND THE UNFOLDED PROTEIN RESPONSE

Chloroplast protein homeostasis is controlled by the balance of *de novo* translation and degradation of unfolded/misfolded/damaged proteins in response to internal and external cues. Because nuclear DNA encodes the vast majority of chloroplast proteins, chloroplast proteostasis also requires nuclear genome coordination for dynamic protein quality control. In particular, a dysfunctional chloroplast defective in proteostasis would require the constant transcription/translation of a suite of nuclear genes encoding proteins involved in protein quality control in order to compensate for the chloroplast dysfunction. For instance, the chloroplasts in the *Arabidopsis yellow variegated 2* (*var2*) mutant, lacking the membrane-bound FtsH2 metalloprotease involved in PSII repair, induce a chloroplast unfolded/misfolded/damaged protein response (cpUPR), primarily due to excess accumulation of oxidized PSII core proteins (Dogra et al., 2019a) (Figure 4A). Similar to the erUPR and the mitochondrial UPR (Walter and Ron, 2011; Naresh and Haynes, 2019), the *ftsH2*-elicited cpUPR leads to upregulation of a group of nuclear transcripts that encode chloroplast proteins involved in protein quality control and ROS detoxification (Dogra et al., 2019a) (Figure 4A). These chloroplast-targeted proteins may compensate for the *ftsH2*-induced deficiency in proteostasis. A comparable molecular phenotype caused by the cpUPR was also observed in *Chlamydomonas* and *Arabidopsis* mutants that exhibited reduced stromal Clp protease activity (Ramundo et al., 2014; Llamas et al., 2017; Kessler and Longoni, 2019). Interestingly, the *clp*-induced chloroplast-targeted chaperones Hsp70 and ClpB3 refold aggregated DXS, the rate-limiting enzyme in the MEP pathway, in *Arabidopsis clp* mutant and WT plants treated with lincomycin, a prokaryotic translation inhibitor (Figure 4B). Increased levels of both chaperones are also observed in *var2* (Figure 4A), indicating that the MEP pathway is likely to be inactivated under chloroplast proteostasis-impairing, ROS-overproducing stress conditions. Moreover, enhanced levels of SA (ICS-dependent) and SA signaling were observed in the *var2* mutant (Figure 4A), linking impaired proteostasis or increased photodamage in chloroplasts to the constitutive activation of SA signaling (Duan et al., 2019). Consistent with this finding, a recent study on PSII

photoinhibition revealed a novel function of SA in protecting PSII and alleviating photoinhibition (Chen et al., 2020).

It is worth mentioning that MEcPP induces erUPR genes in the *ceh1* mutant (Walley et al., 2015), connecting MEcPP-mediated ORS with the erUPR. Similarly,  $^1\text{O}_2$  generation in the *ch1* mutant under moderate light or excess light conditions and in the *flu* mutant after a dark-to-light transition appears to activate the erUPR and the upregulation of nucleus-encoded genes involved in the erUPR (op den Camp et al., 2003; Ramel et al., 2013b; Beaugelin et al., 2020). Consistent with these observations, the erUPR modulates plant resistance to high-light stress to varying degrees. Mild induction of the erUPR in plants treated with a low concentration of tunicamycin (Tm, an inhibitor of protein glycosylation, which thus induces ER stress) protects the plant from photo-oxidative damage under high-light stress. By contrast, a high concentration of Tm, which strongly induces the erUPR, leads to extensive photo-oxidative damage and cell death upon exposure to high-light stress (Beaugelin et al., 2020). Collectively, these findings suggest that  $^1\text{O}_2$  and MEcPP production in chloroplasts stimulate induction of the erUPR, affecting whole-cell stress responses, including proteostasis.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Although photosynthesis is one of the oldest metabolic pathways, it is also known, paradoxically, to be the most sensitive to light. For chloroplast biologists, whose research focuses mainly on stress, the most intriguing products of photosynthesis are the ROS. Because they primarily attack photosystems, aggravating PSII photoinhibition and thus compromising photosynthesis, it is surprising that ROS still cause such problems in plants after almost 2.5 billion years of oxygenic photosynthesis. If photosynthetic ROS only cause photodamage, plants must suffer during their entire lifespan. In this regard, ROS-triggered ORS involved in various physiological processes provides an essential clue to how plants have co-evolved with oxygenic photosynthesis together with ROS. However, our understanding of ROS pathways remains superficial, especially regarding their communication with other well-known signaling pathways triggered by various stress factors. Although the chloroplast is the leading factory producing stress hormones, it is yet unknown how chloroplast ROS signaling pathways interact with SA, JA, and ABA signaling pathways and whether there are signaling hubs that integrate these signaling pathways and regulate them appropriately. Combining “omics” technologies and reverse genetic approaches with conditional ROS and stress hormone biosynthesis/signaling mutants would help us to resolve the complexity of these signaling networks.

Given that chloroplasts rapidly overaccumulate chemically distinct ROS upon exposure to various stress factors, investigating oxidative modification of chloroplast proteins in a non-targeted manner may facilitate the study of sensory proteins involved in ORS. Perhaps various stress factors induce different and distinct oxidation profiles of chloroplast proteins, providing an important clue regarding the primary ROS that plays a dominant role under specific stress conditions. Not only signaling but also detoxification of ROS and lipid peroxides can be

modulated by replacing oxidation-prone residues, such as Trp and Cys, in target proteins. For instance, oxidation-prone Trp residues in PSII core proteins can be substituted in *Chlamydomonas* or model plants to explore the biological significance of these oxidations in the context of photodamage and photoprotection. It would also be inspiring to examine whether ROS-detoxifying enzymes undergo ROS-dependent oxidation, by which one could also modulate their stability and activity.

Like beneficial bacteria, which enhance plant growth and stress tolerance by producing volatile compounds, the endosymbiont chloroplasts also produce volatile compounds that play critical roles in promoting plant growth and stress resistance. The positive nature of volatile apocarotenoids and their electrophilic characteristics already indicate the next goal of this research, i.e., finding the receptor or receptor complex responsible for the physiological responses under volatile-inducible oxidative environmental conditions. The receptor may be present within the chloroplast or the cytoplasm. However, given their versatile impact on abiotic and biotic stress responses, the volatile apocarotenoids may converge with known signaling pathways (e.g., stress hormone signaling pathways). Not only single but perhaps multiple signaling components may sense biologically active concentrations of volatile apocarotenoids to enhance or repress their signaling cascades. Because  $\beta$ CC modulates the MEP pathway and phytohormone-mediated stress signaling, finding its receptor(s) and/or interacting protein(s) may unravel the complexity of  $\beta$ CC-related signaling networks.

Chloroplast-to-nucleus retrograde signaling pathways contribute to plant stress responses, including acclimation and cell death, in response to various stress factors rather than being central signaling pathways. Otherwise, earlier forward genetic screens to find vital components of plant stress responses under abiotic and biotic stress conditions would have revealed chloroplast proteins as central regulators. Instead, chloroplast signaling pathways seem to modulate plant stress responses by utilizing various oxidation-prone signaling components and oxidation-sensitive metabolisms. The genetic and chemical manipulation of their sensitivity toward ROS and redox changes could pave the way for agricultural innovation.

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