Running Title: Oxygen reduction capacity during photosynthesis

Title

Recording true oxygen reduction capacity during photosynthetic electron transfer in Arabidopsis thylakoids and intact leaves

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One sentence summary

Photosynthetically derived H₂O₂ only accumulates at Photosystem I and may trigger cooperation with mitochondria during stress

Abstract

Reactive oxygen species (ROS) are generated in electron transport processes of living organisms in oxygenic environments. Chloroplasts are plant bioenergetics hubs where imbalances between photosynthetic inputs and outputs drive ROS generation upon changing environmental conditions. Plants have harnessed various site-specific thylakoid membrane ROS products into environmental sensory signals. Our current understanding of ROS production in thylakoids suggests that oxygen (O_2) reduction takes place at numerous components of the photosynthetic electron transfer chain (PETC). To refine models of site-specific O_2 reduction capacity of various PETC components in isolated thylakoids, the stoichiometry of oxygen production and consumption reactions, associated with H_2O_2 accumulation, was quantified using membrane inlet mass spectrometry and specific inhibitors. Combined with P700 spectroscopy and electron paramagnetic resonance spin trapping, we demonstrate that electron flow to PSI is essential for H_2O_2 accumulation during light-induced photosynthetic electron transport process. Further leaf disc measurements provided clues that H_2O_2 from PETC has a potential of increasing mitochondrial respiration and CO_2 release.

Introduction

Plants exploit a range of redox balancing and signaling mechanisms to maintain an energetic homeostasis in chloroplasts, particularly important under fluctuating light conditions. These mechanisms alleviate over-excitation and over-reduction of critical photosynthetic electron transfer chain (PETC) components that may generate reactive oxygen species (ROS). Although ROS formation within the photosynthetic apparatus contributes to photodamage, they also function as critical feedback mechanisms and chemical messengers to modify and restore an optimal redox balance. Because ROS formation occurs at the crossroads between photosynthetic damage and regulation, the elucidation of the specific locations and mechanisms of photosynthetically derived ROS generation is critical for understanding the interactions between photodamage and photoprotection. Such knowledge is central for future projects aimed at improving photosynthetic productivity.

Understanding site specific ROS formation within the thylakoid membrane is complicated by the fact that the various components of the PETC participate in several redox reactions, forming a variety of site-specific ROS molecules with varying reactivity and reaction products. For example, during conditions of over-excitation, highly reactive and long-lived chlorophyll triplet states can form and photosensitize molecular oxygen (O_2) to singlet oxygen (1O_2) (Halliwell, Gutteridge 1984). This short-lived and highly reactive form of oxygen is mostly associated with PSII (Durrant, Giorgi et al. 1990, Telfer, Oldham et al. 1999) and leads to rapid peroxidation of proteins, lipids and nucleotides. On the other hand, electron transfer within the PETC can result in the reduction of O_2 to form the superoxide anion ($O_2^{\bullet-}$). This reaction was originally described by Mehler (Mehler 1951) and occurs primarily at the acceptor side of PSI (Mehler 1951, Furbank, Badger 1983). To counter this

reaction, the catalyst superoxide dismutase (SOD) enzymatically dismutates two $O_2^{\bullet -}$ molecules into the oxidant H_2O_2 , with a release of one O_2 . Although H_2O_2 is relatively stable in biological systems, further reduction of H_2O_2 can result in the formation of highly destructive hydroxyl radicals ($HO^{\bullet}$). To minimise potential damage from such destructive molecules, strong ROS scavenging systems comprising peroxiredoxins, ascorbate and ascorbate peroxidase enzymes (Asada 2006) operate in both the thylakoid membrane and in the stroma. They facilitate the complete enzymatic two-electron reduction and protonation of H_2O_2 , thereby yielding two water molecules and completing an electron pathway described as the water-water cycle (WWC) (Asada 1999). Despite the antioxidant systems that can efficiently quench ROS, a growing body of evidence suggests that photosynthetically derived ROS such as H_2O_2 act in environmental sensing (Mubarakshina, Ivanov 2010) and intercellular relay of information (Fichman, Miller et al. 2019) or function directly as a retrograde signal exported from the chloroplasts to the nucleus (Gollan, Aro 2020, Exposito-Rodriguez, Laissue et al. 2017).

Numerous strategies to measure site-specific ROS formation in PETC have been developed and applied over several decades. The resulting literature suggests that in addition to the Mehler reaction at PSI, $O_2^{\bullet -}$ and H_2O_2 can form within the PETC at heavily reduced PSII (Tiwari, Pospisil 2009), the PQ pool (Khorobrykh, S. A., Ivanov 2002, Mubarakshina, Ivanov 2010, Mubarakshina Borisova, Kozuleva et al. 2012, Khorobrykh, Sergey A., Karonen et al. 2015), plastid terminal oxidase (PTOX) (Heyno, Gross et al. 2009) and Cyt-*b₆f* complex (Baniulis, Hasan et al. 2013). The current work aimed at understanding whether these different sites can form stable H_2O_2 pools capable of functioning as secondary messengers in guiding plant acclimation according to the environmental cues. To this end, we measured the true capacity of oxygen reduction/consumption at PSII, PQ-pool, and Cyt-*b₆f* in proportion to the PSII water oxidation rates.

A new approach to site-specific ROS measurements - It is necessary to revisit site-specific ROS formation in the thylakoid membrane, incorporating stronger controls, higher precision and with the capacity to discriminate O_2 reduction and true H_2O_2 accumulation. Incorporating lessons from our recent finding of the failure of DNP-INT to completely block electron transfer to PSI (Fitzpatrick, Aro et al. 2020), we have taken a methodical three-step approach to re-examine ROS formation and accumulation in isolated thylakoids of *Arabidopsis thaliana* (hereafter Arabidopsis) and have then tested our conclusions in-vivo.

As the first step, to avoid artefacts from any residual activity of the PSI-Mehler reaction, we measured P700 redox kinetics under experimental conditions to verify that each PETC inhibitor (Fig. 1, as described schematically) completely blocked the re-reduction of oxidized P700 ($P700^+$). As the second step, we took advantage of the fact that isolated thylakoids lack the stroma and terminal electron acceptor $NADP^+$, and thus the electrons derived from oxidation of H_2O at PSII are transferred terminally to O_2 via the PETC. The overall process can be dissected into partial reactions (Asada 2006) (Fig. 1, Reactions 1 – 4). These partial

reactions can be estimated from the stoichiometry of O₂ produced and O₂ consumed at each step. Reaction (1) -The oxidation of two H₂O molecules at PSII releases one O₂ and four electrons into the PETC. Reaction (2) –The four electrons reduce four O₂ molecules to O₂^{-•}. Reaction (3) - The four O₂^{-•} dismutate or decompose (rapidly) into two H₂O₂ molecules, reforming two molecules of O₂. Reaction (4) - A complete degradation of two H₂O₂ further yields one molecule of O₂ and two H₂O, the latter reforming the H₂O originally oxidized at PSII, resulting in a net O₂ flux of zero. At each of these 4 steps the stoichiometric ratio of O₂ produced to O₂ consumed varies, which we describe as the O₂ flux ratio (Fig. 1, O₂ Flux Ratios). The O₂ flux ratio was quantified with MIMS by separating the two events from each other *i.e.* the rate of PSII H₂O splitting reactions were measured by the production rate of

the naturally abundant O₂ isotopologue (Fig. 1, ¹⁶O₂, blue font), whilst O₂ reduction reactions were determined by the consumption rate of the artificially enriched heavy O₂ isotopologue (Fig. 1, ¹⁸O₂, red font).

The key advantages of the first and second steps of our approach, compared to measuring net O₂ fluxes polarographically, are that (1) no assumptions are required to determine the number of electrons entering the PETC, (2) the 1:1 ratio of Reaction 4 is measurable, even if H₂O₂ is catalytically decomposed and (3) it is possible to clearly observe when O₂ was consumed in the absence of electron transport, *i.e.* to observe the 0:1 stoichiometry described in Reaction 5 (Fig. 1), which results from lipid/protein peroxidation reactions such as those resulting from ¹O₂ formation. As the third step in our approach, Electron Paramagnetic Resonance (EPR) spin-trapping was applied to thylakoid samples under similar experimental conditions. This enabled us to directly infer the ROS formed at each location in the PETC, to support the observations and conclusions made with the MIMS data.

Results

Controlling for ROS artefacts resulting from only partial inhibition of PSI - As described in Figure 1, application of specific inhibitors enabled systematic segmentation of the PETC into discrete components. It was then possible to measure the site-specific ROS formation of each component with MIMS or EPR. However, to avoid artefacts from an active PSI-Mehler reaction during sample illumination, the efficacy of each inhibitor to completely block re-reduction of oxidised P700 (P700⁺, special chlorophyll pair in PSI) was measured via P700 spectroscopy with a Dual PAM, under emulated experimental conditions. The redox measurement and quantification of P700 in Dual PAM is based on strong absorption characteristics of oxidised P700 between 800 to 840 nm. A difference of transmittance between the 875 nm and 830 nm in darkness shows completely reduced P700. The calibrated kinetic signal of P700 is represented as $\Delta I/I \times 10^{-3}$ units. The following inhibitors were tested and used: (1) DCMU, as an inhibitor of PQ pool reduction, enabled PSII to be heavily reduced (Witt, Rumberg et al. 1968), (2) DBMIB, as an inhibitor of PQ oxidation by Cyt-*b₆f*, enabled complete reduction of PSII and the PQ pool (Bauer, Wijnands 1974) and (3) HgCl₂, as an inhibitor of plastocyanin (PC) function, enabled reduction of PSII, PQ pool and Cyt-*b₆f* (Kimimura, Katoh 1972). Historically, DNP-INT has been used to block reduction of Cyt-*f* whilst enabling interaction of the low potential chain of Cyt-*b₆f* complex with plastoquinone, considered necessary for the formation of O₂^{-•} within the PQ-pool (Khorobrykh, S. A., Ivanov 2002, Mubarakshina, Ivanov 2010). However, in an attempt to maintain such dynamics between the PQ pool and Cyt-*b₆f* in isolated thylakoids and leaf discs, we observed that DNP-INT failed to completely block the reduction of PSI (Fitzpatrick, Aro et al. 2020). Hence, HgCl₂ was considered the best available alternative. Although HgCl₂ has been reported to have multiple inhibitory effects on photosynthetic apparatus

(Carpentier 2001), we observed strong PSII activity in isolated thylakoid membranes in the presence of HgCl₂ under both growth light and high light (see Supplemental Fig. S1). Indeed, to avoid measuring artefacts with EPR or MIMS from a still active PSI, we systematically confirmed that each inhibitor could completely block reduction of oxidised PSI (achieved through application of a background FR light) from receiving electrons sourced at PSII following the application of strong actinic light pulses (ST and MT) (Fig. 2).

Freshly prepared isolated thylakoid (Fig. 2A) and intact leaf (Fig. 2B) samples from Arabidopsis were incubated in darkness with 10 μ M DCMU, 10 μ M DBMIB or 2 mg ml⁻¹ HgCl₂ (we found HgCl₂ toxic to intact leaves so this inhibitor was applied only for isolated thylakoid samples). As negative controls for blocking PSI activity, 10 μ M Methyl Viologen (MV, catalyst of O₂ reduction at PSI acceptor side) and untreated control samples were also measured. During the preceding dark incubation, a single saturating actinic light pulse (SP) revealed an overall capacity of each sample to rapidly oxidise P700 followed by fast re-reduction in all samples except for the HgCl₂-treated thylakoids. Subsequent continuous background FR light (128 μ mol photons m⁻² s⁻¹) preferentially oxidized P700 to P700⁺. To activate electron flow from PSII to PSI, a 50 μ s single turnover (ST), followed by a 50 ms multiple turnover (MT) pulse of actinic light (10 000 μ mol photons m⁻² s⁻¹) was superimposed over the FR background. The electrons released from water by PSII into the

PETC by these actinic light pulses transiently reduced $P700^+$ in both sets of untreated control samples (Fig. 2, A and B, black traces), evidenced by the pulse length dependent decrease, or 'dip' of the $P700^+$ signal, before being fully re-oxidised by the continuous FR background light (Tiwari, Mamedov et al. 2016). In contrast, no transient dip was observed in the $P700^+$ signal when thylakoid samples were treated with DCMU, DBMIB or $HgCl_2$ (Fig. 2A, red, blue and pink color traces), or in leaves treated with DCMU or DBMIB (Fig. 2B, red and blue traces). This confirmed that these inhibitors blocked all electron transport from PSII to PSI. Uniquely, $HgCl_2$ blocked the re-reduction of $P700^+$ in isolated thylakoid samples following the actinic light pulse in the initial dark phase of the measurement (Fig. 2A, pink trace). This was consistent with $HgCl_2$ targeting the function of PC, which was then severely restricted in its capacity to deliver electrons to $P700^+$ throughout the rest of the measurement. Samples infiltrated with MV (Fig. 2, A and B, green traces) exhibited clear actinic light dependent dips. However, due to the accelerated rate of re-oxidation produced by the artificial PSI acceptor, and catalyst of $O_2^{\cdot-}$ formation, the 'dips' following ST and MT pulses were 'narrowed' compared to the control samples (Fig. 2, A and B, black traces,) in which electrons took longer to find an acceptor.

O₂ flux ratio measured with MIMS positively identified H₂O₂ accumulation in thylakoid samples - Freshly prepared thylakoid samples were illuminated at a relatively low light intensity, equivalent to growth light (GL) ($120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), to minimise 1O_2 formation and to verify that H_2O_2 accumulation results in a 1:2 O₂ flux ratio (Fig. 1) in MIMS measurements (Fig. 3, A-G). To ensure that the O₂ flux ratios were calculated based on light dependent O₂ fluxes, the background dark rate of O₂ consumption (also observed in (Furbank, Badger 1983)) was offset to zero. Control samples (Fig. 3A) produced O₂ in a relatively stable manner across the three minutes of illumination, at a rate of approximately $2.5 \mu\text{mol mg chl}^{-1} \text{h}^{-1}$. The simultaneous rate of O₂ consumption was less stable, exhibiting a steady decrease in the integrated rate over the three minutes of illumination. However, the initial O₂ consumption rate of approximately $5.0 \mu\text{mol mg chl}^{-1} \text{hr}^{-1}$ was double the initial O₂ production rate, resulting in an O₂ flux ratio of 1:2, consistent with accumulation of H_2O_2 (Fig. 1). The addition of catalase ($700 \text{ unit per ml}^{-1}$) to rapidly degrade any H_2O_2 formed did not drastically affect the rate or stability of O₂ production (Fig. 3B). However, it decreased the absolute rate of O₂ consumption so that it matched the O₂ evolution rate, resulting in the 1:1 O₂ flux ratio expected in the absence of H_2O_2 accumulation (Fig. 1). It is clear that when H_2O_2 accumulates, the rate of O₂ consumption is greater than the rate of O₂ evolution (Fig. 3, A and C) (red line is a greater magnitude than the blue line and net rate shown with a grey line is negative). In contrast, when the accumulation of H_2O_2 is excluded by the addition of catalase (Fig. 3, B and D) or in samples excluding PSI function (Fig. 3, E-G), we clearly observed that O₂ consumption cannot significantly exceed O₂ production, as any H_2O_2 formed was immediately decomposed, re-releasing the $^{18}O_2$. As a positive control for H_2O_2 accumulation, and to simultaneously test the sample's acceptor side limitation at PSI, $10 \mu\text{M}$ MV was added to thylakoid samples. The O₂ evolution

rate increased to $12.5 \mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$ and the initial O_2 consumption rate of $25.0 \mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$ reproduced the O_2 flux ratio of 1:2 observed in control samples (Fig. 3C, observe change in y-axis scale). This result verified that control samples illuminated at GL were PSI acceptor limited, therefore all components of the thylakoid membrane were heavily reduced during illumination. The addition of catalase to MV-treated thylakoid samples (Fig. 3D) replicated the effects of catalase added to control thylakoids, reproducing a 1:1 O_2 flux ratio with no effect on the rate of O_2 production. The strong negative O_2 consumption rate following illumination, both in control and amplified in MV treated samples, suggested that the declining O_2 consumption rate during illumination was a product of $^{18}\text{O}_2$ release from an accumulated pool of H_2O_2 . This was supported by the observation that the sum of the O_2 consumption rate at the end of the light period and the negative rate at the beginning of the dark period approximated the initial O_2 consumption rate in untreated control and MV samples (for a more detailed explanation of interpreting time resolved gas flux transients, and how the negative O_2 consumption burst has been

interpreted as the decomposition of an accumulated H_2O_2 pool, refer to Supplemental Fig. S2). In the summary bar chart of the O_2 flux ratios (Fig. 3H) it can be seen that the control and MV treated samples exhibited an O_2 flux ratio of 1:2. Conversely, in samples where catalase precluded H_2O_2 accumulation an O_2 flux ratio of 1:1 was observed. Hence, MIMS was able to discriminate between isolated thylakoid samples in which H_2O_2 was accumulated from those in which the H_2O_2 was rapidly decomposed.

MIMS confirms that O_2 reduction at PQ-pool and Cyt-*b₆f* cannot accumulate H_2O_2 - To compare the site-specific capacity of heavily reduced PETC components upstream of PSI to generate ROS and accumulate a pool of H_2O_2 in-vitro, we measured the O_2 flux ratios of freshly isolated thylakoid samples incubated with 10 μM DCMU, 10 μM DBMIB or 2 mg ml^{-1} HgCl_2 . Based on the results of MV addition to isolated thylakoids, discussed above, we were confident that components within the PETC were heavily reduced at GL irradiance. Incubation with DCMU clearly impaired all O_2 production (Fig. 3E) and although noisy, O_2 consumption was minimal. In contrast, samples incubated with DBMIB exhibited strong O_2 production by water oxidation at the initiation of illumination, in the absence of commensurate O_2 consumption. This resulted in an initial O_2 flux ratio of 1:0 (Fig. 1, Reaction 1), consistent with DBMIB accepting electrons from PSII as reported previously (Lozier, Butler 1972). The rate of O_2 production peaked, presumably as available DBMIB was fully reduced, and then slowed as O_2 consumption became the primary electron sink (more evidence that samples were acceptor-limited). The steady-state rate of O_2 production and consumption of approximately 1.0 $\mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$ (Fig. 3F) comprised an O_2 flux ratio of 1:1. This ratio matched the addition of catalase to control and MV treated thylakoid samples. According to the results of a DBMIB concentration response curve, the integrated area of the O_2 evolution peak was DBMIB concentration dependent (r^2 of 0.99) whilst the steady-state rate following the peak was DBMIB concentration independent (Supplemental Fig. S3). This result thus suggested that DBMIB was not acting as a redox intermediate in the steady-state O_2 consumption pathway. Incubating thylakoids with HgCl_2 (Fig. 3G) produced a very similar PSII activity as that in DBMIB samples, exhibiting a steady-state rate of O_2 production approximately 1.0 $\mu\text{mol O}_2 \text{ mg chl}^{-1} \text{ h}^{-1}$ and a 1:1 O_2 flux ratio. Significantly, the lack of a large initial peak in O_2 production supported the conclusions drawn from the DBMIB concentration response curve (Supplemental Fig. S3). The small initial peak in O_2 production observed with HgCl_2 was also evident in control samples with and without catalase (Fig. 3, A and B) but absent from DCMU curves, suggesting it was the product of reducing a small intrinsic pool of acceptors between PSII and PSI, likely the PQ pool.

The fact that HgCl_2 and DBMIB samples exhibited almost identical steady-state rates and a 1:1 O_2 flux ratio at GL supported the contention that a pathway to O_2 reduction operates within the thylakoid membrane. Whilst this pathway upstream of PSI exhibited a smaller capacity to reduce O_2 compared to the PSI-Mehler reaction, it clearly supports PSI independent steady-state turnover of PSII. PTOX may be a candidate to explain such a

pathway, reducing O₂ to H₂O with reductant from PQH₂ (Cournac, Josse et al. 2000). In an effort to control for PTOX function we repeated DBMIB measurements in the presence of the widely used PTOX inhibitors *n*-propyl gallate or octyl gallate (Cournac, Josse et al. 2000). Unexpectedly in our measurements, both treatments enhanced O₂ production (PSII activity) and O₂ consumption, even in the presence of DCMU (Supplemental Fig. S4, with short discussion of these experiments). We did not investigate this result further, concluding that based on analysis of the P700 kinetics of isolated thylakoids (Fig. 2A) and the O₂ Flux ratios of thylakoid samples (Fig. 3H), H₂O₂ can only accumulate in the thylakoid membrane if electrons are able to reach PSI.

Increasing the redox and excitation pressure highlighted separate ROS pathways within the thylakoid membrane in-vitro - To test the impact of increased excitation and redox pressure on the results described above, we repeated all MIMS measurements with inhibitors using high light illumination (HL, 900 μmol photons m⁻² s⁻¹ (Fig. 4). The untreated control samples increased rates of O₂ production and consumption to approximately 5 and 10 μmol O₂ mg chl⁻¹ h⁻¹, respectively (Fig. 4A), whilst the 1:2 O₂ flux ratio was maintained. We assured that relatively low rates observed were a result of the lack of electron acceptors in isolated thylakoids (no externally added ferredoxin (Fd)), and the absence of extra SOD in our experiments. Contrasting to untreated control thylakoids, the DCMU response was markedly different at higher illumination. The dosage required to fully impair PSII function increased to 50 μM and a strong light dependent, catalase independent, O₂ consumption was observed at a rate of approximately 1.5 μmol O₂ mg chl⁻¹ h⁻¹ (Fig. 4, B and F), consistent with previously published observations (Khorobrykh, S. A., Khorobrykh et al. 2011). The O₂ flux ratio of 0:1 in DCMU treated thylakoids was consistent with activity of Reaction 5 (Fig. 1), suggesting peroxidation of lipids and membranes via formation of ¹O₂ and/or organic peroxides (Khorobrykh, S. A., Khorobrykh et al. 2011), in the absence of water splitting at PSII. Addition of catalase had no effect on the measured rates (Fig. 4F) confirming that H₂O₂ accumulation was not behind the observed O₂ consumption. This is in line with findings, based on the use of the fluorescent biosensor HyPer2, that PSII cannot generate H₂O₂ in the presence of DCMU (Exposito-Rodriguez, Laissue et al. 2017). Increased irradiance also affected the DBMIB and HgCl₂ treatments. Steady-state O₂ production by DBMIB treated samples increased by approximately 50%, from 1.0 up to 1.5 μmol O₂ mg chl⁻¹ h⁻¹ (Fig. 4C), whilst it increased threefold in the HgCl₂ samples, from approximately 1.0 to 3.0 μmol O₂ mg chl⁻¹ h⁻¹ (Fig. 4D). However, O₂ consumption rates in both treatments increased by a larger relative amount than O₂ production, shifting the apparent O₂ flux ratio towards 1:2 (Fig. 4E). Although, based on the O₂ flux ratio, this suggested that H₂O₂ was accumulating within the thylakoid membrane, we observed that the rate of O₂ consumption in DBMIB samples was insensitive to catalase (Supplemental Fig. S5 A, we could not measure HgCl₂ samples with catalase due to the toxicity of Hg²⁺ for the catalase enzyme). We suspect that the isolated thylakoid samples experience strong acceptor side limitation at PSII. This was exacerbated

Figure 4. Integrated rates of $^{16}\text{O}_2$ production and $^{18}\text{O}_2$ consumption by isolated thylakoid samples, measured simultaneously at $900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with MIMS. Illumination of samples represented by yellow bar with grey representing darkness. A, untreated control, B, $50 \mu\text{M}$ DCMU C, $10 \mu\text{M}$ DBMIB D, 2 mg ml^{-1} HgCl_2 . O_2 consumption rates increased more than O_2 production rates in all inhibited samples, compared to measurements at $120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. F, $50 \mu\text{M}$ DCMU + Catalase suggests $^1\text{O}_2$ formation as a likely reason for increased O_2 consumption, subsequently this curve was subtracted from both DBMIB and HgCl_2 curves resulting in G, DBMIB-(DCMU + Catalase) and H, HgCl_2 -(DCMU + Catalase). E, Plotting O_2 production to O_2 consumption ratios from all curves highlights that subtraction of O_2 consumption associated with peroxidation of lipids, proteins and membranes by $^1\text{O}_2$ resulted in the return of O_2 flux ratios that exclude the accumulation of H_2O_2 . All curves are an average of minimum three representative replicates plotted with standard error. All measurements of isolated thylakoids were performed in measurement buffer containing the uncoupler NH_4Cl (5mM).

by the higher irradiance which increased the probability of chlorophyll triplet states and therefore $^1\text{O}_2$ formation. The $^1\text{O}_2$ associated lipid/membrane peroxidation was enhanced by the HL treatment, resulting in a strong background rate of O_2 consumption not associated with electron transport or $\text{O}_2^{\bullet-}$ formation, previously defined as DCMU insensitive O_2 consumption (Furbank, Badger 1983).

This interpretation was supported when we tested MV+catalase at high light, in which a small catalase insensitive O_2 consumption was also observed (Supplemental Fig. S5 B). In order to account for the contribution of $^1\text{O}_2$ formation in calculating the absolute O_2 flux ratios of DBMIB and HgCl_2 samples, the DCMU + catalase rates were directly subtracted

from the DBMIB and HgCl₂ results (Fig. 4, G and H). Resultant curves from both DBMIB and HgCl₂ treatments were almost identical to those measured at GL, excluding the slight increase in steady-state gas fluxes. Both exhibited an O₂ flux ratio of approximately 1:1 (Fig. 4E), further supporting that H₂O₂ cannot accumulate within the thylakoid membrane at either PSII, the PQ pool or Cyt-*b₆f* complex. This conclusion was further reinforced by the insensitivity to catalase of the HL enhanced O₂ uptake of DBMIB treated thylakoid samples (Supplemental Fig. S5 A).

EPR spin trapping data, consistent with conclusions from in-vitro MIMS measurements, supports the function of PTOX - To test conclusions relating to ROS formation in the PETC based on MIMS measurements, we next applied EPR spin trapping to isolated thylakoid samples. We used DIPPMPO, a more lipophilic spin trap with higher sensitivity and greater adduct stability for O₂^{-•} (DIPPMPO-OOH), OH^{-•} (DIPPMPO-OH), and carbon-centred adducts (DIPPMPO-R) than other commonly used spin traps like DMPO (Villamena 2017). Isolated thylakoids were incubated with DIPPMPO in darkness for five minutes, before sample illumination with actinic light for three minutes at 150 μmol photons m⁻² s⁻¹. Samples were then centrifuged and the supernatant transferred into an EPR capillary for measurement, while the pellet was discarded. To compare the relative capacity for ROS formation at different locations within the PETC compared to untreated controls, the inhibitors DCMU and HgCl₂ were applied across a series of measurements with MV included as a positive control for enhanced O₂^{-•} adduct formation. Based on our MIMS results on redox activity of DBMIB (Fig. 3F and Fig. 4, C and G), we excluded DBMIB from EPR measurements as it is also known to alter the redox potential of the Iron-sulphur clusters of PSI and Cyt- *b₆f* complex (Malkin 1981), thus possibly producing artefacts and interference in EPR spin trapping of ROS.

To control putative background EPR signals, potentially arising from DIPPMPO incubation with isolated thylakoids and the inhibitors, the EPR response of each sample was measured after five minutes of dark incubation. No EPR signal was produced in darkness (Fig. 5A). Illumination of the spin trap with control thylakoids generated a clear spin trap -ROS adduct signal (Fig. 5B, black trace) which increased 2-3 fold through addition of MV (Fig. 5B, purple trace). We performed a simulation fitting of the experimental EPR spectra to figure out what proportion of different radicals are present in it. Auto simulation was performed by Winsim application using previously reported values of hyperfine coupling constants for DIPPMPO adducts (Chalier, Tordo 2002), setting a 1% limit for g-value and hyperfine coupling constants but enabling automatic adjustment for other parameters during fitting. The best fit simulated spectrum of DIPPMPO radical is obtained (Fig. 5C) for an experimental spectrum of untreated control thylakoids (Fig. 5B, upper trace). It contained approximately 73% O₂^{-•} adduct (DIPPMPO-OOH), 18 % OH^{-•} (DIPPMPO-OH) and 9% carbon-centred adduct (DIPPMPO-R). Variation of 5 – 10% was observed in the contribution of superoxide and hydroxyl adducts between biological replicates.

The addition of DCMU decreased the measured spin-trap signal by 90% compared to untreated controls (Fig. 5B). A very weak signal in DCMU treated thylakoids comprised approximately 65% carbon centred radicals and only small contributions were observed from $\text{O}_2^{\cdot-}$ and $\text{OH}^{\cdot}$ adducts (Fig. 5B, blue trace). This was consistent with the 0:1 O_2 flux ratio (Fig. 1, Reaction 5) observed in MIMS data (Fig. 4B) and the peroxidation of lipids and membranes via formation of $^1\text{O}_2$ and/or organic peroxides (Khorobrykh, S. A., Khorobrykh et al. 2011). The addition of HgCl_2 reduced the adduct signal up to 95% compared to the untreated control samples (Fig. 5B, green trace), despite MIMS data showing that HgCl_2 only slowed PSII O_2 evolution, and associated O_2 consumption, by approximately 40 % (Fig. 3G and 4H). Whilst the O_2 dynamics strongly suggest that electrons produced through oxidation of water at PSII were accepted by O_2 , the lack of $\text{O}_2^{\cdot-}$ adduct formation infers enzymatic reduction of O_2 to water, without formation of ROS. Such a pathway is reminiscent of the flavodiiron proteins functional at the PSI acceptor side in lower-order phototrophs and

gymnosperms (Ilík, Pavlovič et al. 2017), and makes it conceivable that PTOX performs a similar function in flowering plants. The data is also consistent with function of the theorised PQH₂/PQH⁻ mechanisms for formation and quenching of O₂^{-•} (Khorobrykh, Sergey, Tyystjärvi 2018). However, spin-traps are notorious for their inefficiency at penetrating the membranes, making this data difficult to interrogate further. Despite this, the suppression of the EPR spin-trap signal (Fig. 5B), in conjunction with the 1:1 O₂ flux ratio measured with MIMS (Fig. 3G and 4H), rules out the accumulation of H₂O₂ in HgCl₂ treated thylakoid samples. As HgCl₂ enabled the reduction of all components upstream of PSI, these data offer robust evidence that stable accumulation of H₂O₂ requires PSI activity.

Detecting photosynthetic H₂O₂ accumulation via O₂ stoichiometry in leaf discs - To test the applicability of our in-vitro findings we developed a MIMS methodology to estimate O₂ flux ratios in-vivo (Arabidopsis leaf discs), under similar conditions to those tested with isolated thylakoids. As the primary terminal electron acceptor in leaves is CO₂ and mitochondrial respiration is a large contributor to O₂ consumption, it was not possible to simply divide the gross O₂ production rate by the gross O₂ consumption rate. Alternatively, we estimated the O₂ flux ratio associated specifically with the steady-state WWC determined in the absence of photorespiration. Assuming a 1:1 ratio between ¹⁶O₂ evolution and ¹³CO₂ (artificially enriched) fixation by Rubisco during oxygenic photosynthesis, we determined the difference between the production of electrons at PSII and the consumption of electrons by the Calvin-Benson-Bassham (CBB) cycle. This difference provided an upper limit of ¹⁶O₂ production specifically linked to the WWC. The rate of ¹⁸O₂ consumption associated with the Mehler reaction was then determined by subtracting the rate of ¹²CO₂ produced by mitochondrial respiration, from the gross rate of ¹⁸O₂ consumption (assuming a 1:1 ratio between CO₂ production and O₂ consumption during aerobic mitochondrial respiration). By simultaneously measuring the fluxes of four stable isotopes, representing isotopologues of both O₂ and CO₂, we could estimate the steady-state rate of O₂ production and O₂ consumption associated specifically with the WWC and hence, estimate the in-vitro and in-vivo O₂ flux ratio according to the scheme in Figure 1.

Arabidopsis leaf discs (12.5 mm in diameter) were floated overnight in near darkness on water containing 10 μM DCMU, 10 μM MV or 10 μM DBMIB (or plain water – untreated control), then sealed into the MIMS cuvette and purged with air scrubbed of all ¹²CO₂ (Soda Lime, Li-Cor USA). To this 3% ¹³CO₂ and 2% ¹⁸O₂ were injected by volume (total O₂ approximately 21%). After a few minutes darkness to ensure isotopic equilibrium throughout the sample, data acquisition was initiated. An illumination protocol of three minutes darkness, five minutes at GL, five minutes at HL and three minutes darkness was applied. In the data, all samples exhibited an apparent ‘peak’ of ¹⁸O₂ production during transition from GL to HL, and ¹⁸O₂ uptake following transition from HL to darkness. This trend was also observed in the blank control measurements (Supplemental Fig. S6) indicating that it was an artefact, potentially a light effect on the membrane. To avoid this

artefact and emphasise the steady-state results in the main panels, the integrated gas flux rates versus time (Fig. 6, A, B, D and E) are displayed with breaks in the x-axis. For reference, the inset figures display the full curves. All rates used to calculate the O₂ flux ratios (Fig. 6F) are based on the averaged steady-state fluxes measured from minimum three biological replicates. These values are presented as a table in the supplementary data (Supplemental Tab. S1).

Figure 6. The integrated rates versus time of O₂ and CO₂ fluxes from intact leaf discs measured at three different light conditions. Rate versus time plots of A, control, B, MV, D, DBMIB, E, DCMU infiltrated leaf discs. Inset shows complete curves and the main figure highlights steady-state rates across the dark and two light intensities. GL = 120 and HL = 900 μmol photons m⁻² s⁻¹ respectively. For direct comparison, C, shows CO₂ efflux rates (mitochondrial respiration) from the four treatments. F, O₂ flux ratio associated with Mehler reaction calculated as described in text. All curves average of minimum three representative replicates plotted with standard error. The apparent mismatch between activation of light and photosynthetic activity is a result of the integration of rates over time required to minimise noise and allowing a focus on steady state rates.

Untreated control leaf discs (Fig. 6A) performed as expected in a high CO₂ environment. Rates of O₂ production and CO₂ fixation (Fig. 6, blue and green curves) were strongly correlated across all irradiances. The ¹²CO₂ efflux generated by mitochondrial respiration apparently decreased in correlation with increasing irradiance (Fig. 6, A-E, pink line, Fig. 6C, highlighted by purple line). This result was likely a product of CO₂ re-fixation (Busch, Sage et al. 2013), as such dark respiration was assumed to be constant when calculating the in-vivo

O₂ flux ratio from untreated control discs, as concluded by (Farquhar, Busch 2017). The rate of O₂ production providing electrons used specifically in the Mehler reaction of control discs was estimated at 0.4 ± 0.3 and $0.7 \pm 0.6 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ at GL and HL respectively. This matched the estimated rate of Mehler O₂ consumption of 0.4 ± 0.1 and $0.6 \pm 0.4 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ at GL and HL.

The resulting in-vivo O₂ flux ratio of approximately 1.0 (GL = 1.0 ± 0.1 , HL = 1.1 ± 0.1) discounted accumulation of H₂O₂, as expected with an elevated CO₂ atmosphere. Despite differences in methodology, these rates compare well with previous MIMS based estimates of Mehler specific O₂ consumption under similar conditions (Mubarakshina, Ivanov 2010).

The DBMIB treatment completely impaired ¹³CO₂ fixation (Fig. 6D, green line) and had no effect on ¹²CO₂ efflux (Fig. 6C, black line). However, light dependent O₂ production was evident and it correlated with an increase in the rate of O₂ consumption above that required for mitochondrial respiration. This result supports the function of an O₂ reduction pathway within the thylakoid membrane, observed in-vitro, and the calculated O₂ flux ratio of approximately 1.0 (GL = 1.1 ± 0.4 and HL = 0.9 ± 0.3) reinforced the in-vitro conclusions that H₂O₂ cannot accumulate within the reduced PQ pool. The DBMIB dependent O₂ production ‘peak’ was not observed in the leaf discs, potentially due to chlororespiration during dark incubation preceding the measurements that may have fully reduced all DBMIB in the samples before illumination. Contrasting the earlier experiments, DCMU infiltration was not completely effective, although it severely impaired O₂ production during illumination (Fig. 6E). However, this O₂ production was matched by commensurate CO₂ fixation during illumination (GL = 0.1 ± 0.06 , HL = $0.2 \pm 0.2 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Significantly, the illuminated O₂ consumption rate doubled from a dark rate of $0.4 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ to approximately $0.8 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$. In the absence of any change to the respiratory CO₂ efflux (Fig. 6C, green trace) and with all PSII activity accounted for by CO₂ fixation, the light dependent O₂ consumption was most likely a product of ¹O₂ formation and associated peroxidation of lipids, proteins and membranes. The calculated O₂ flux ratio of zero (Fig. 6F) was in-line with our in-vitro MIMS and EPR results and in agreement with published literature that H₂O₂ cannot accumulate at PSII in the presence of DCMU (Exposito-Rodriguez, Laissue et al. 2017).

As a final positive control to test the methodology in estimating H₂O₂ accumulation, we infiltrated leaf discs with MV. In leaf discs MV competes with CO₂ fixation reactions for reductant at PSI, impairing rates of CO₂ fixation compared to untreated control discs. The accumulation of ATP that would otherwise be used in the CBB cycle limits the supply of phosphate for further ATP production. The subsequent impairment of ATP-synthase results in the accumulation of a strong proton gradient across the thylakoid membrane, which impairs O₂ production and likely results in a counterintuitively reduced PQ pool as previously described (Shapiguzov, Nikkanen et al. 2020). The illuminated rate of O₂ consumption in MV treated leaf discs was approximately twofold the rate of O₂ production,

suggesting the accumulation of H₂O₂ (Fig. 6B). However, in darkness MV approximately doubled the ¹²CO₂ efflux rate associated with mitochondrial respiration compared with all other samples (Fig. 6C, orange line), as observed by (Scarpeci, Valle 2008). Uniquely for the MV treated samples, the ¹²CO₂ efflux rate was further enhanced by illumination. Assuming this was due to mitochondrial respiration with a 1:1 respiratory quotient accounted for a significant portion of the increase in O₂ consumption. This observation was only possible due to our application of the ¹³CO₂ offset method, which enabled an accurate O₂ flux ratio of 0.7 ± 0.05 to be determined at both GL and HL. This ratio supports the formation and accumulation of some photosynthetically derived H₂O₂, as more O₂ was consumed than produced by PSII. However, the ratio also suggests that ROS scavenging components associated with the WWC were able to manage approximately half of the H₂O₂ produced in the presence of MV, at the impaired rates of PSII activity. Although the signal to noise ratio of this in-vivo method could not discriminate subtle events, the in-vivo data broadly supported the in-vitro conclusions that 1) H₂O₂ could not accumulate at PSII 2) an O₂ reduction pathway operates in the presence of DBMIB which precludes the accumulation of H₂O₂. In addition, application of ¹³CO₂ to discriminate mitochondrial respiration from CO₂ fixation by the CBB cycle unexpectedly revealed a light dependent stimulation of mitochondrial respiration in MV treated leaf discs, which we examined further.

Functional evidence suggests that H₂O₂ accumulation induces cooperation between chloroplasts and mitochondria - Infiltration with MV resulted in a light dependent increase in mitochondrial ¹²CO₂ efflux. This respiratory ‘burst’ occurred after the light dependent increase in ¹⁸O₂ consumption (Fig. 6B, compare the red and pink curves, note that apparent ‘early’ light response in data is an artefact from integrating data points over 30 seconds, all gas fluxes were integrated equally). To further probe this result we vacuum infiltrated leaves with catalase and with rotenone (inhibitor of mitochondrial Complex-I). Neither compound affected the gas exchange trends of ¹²CO₂ efflux in comparison with untreated control (infiltrated with water) discs (Supplemental Fig. S7, compare blue, pink and black curves). When leaves were infiltrated with MV + rotenone, samples exhibited a similar light induced respiratory burst of ¹²CO₂ efflux as the standard MV infiltrated leaves (Fig. 6C, compare the orange curve to the red curve in Supplemental Fig. S7). However, leaves infiltrated with MV + catalase shared dynamics with DCMU and DBMIB infiltrated discs, showing no light dependent burst of ¹²CO₂ efflux (Fig. 6C, compare green and black curves with the green curve of Supplemental Fig. S7). Therefore, the light dependent ¹²CO₂ burst was only apparent following infiltration with MV, was not affected by rotenone but was blocked by catalase. We speculate this may be evidence of H₂O₂ signaling to the mitochondria (Cui, Brosché et al. 2019), which could relate to ‘malate cycling’ (Zhao, Yu et al. 2020), possibly evidence that the mitochondria are primed to process an influx of malate during a prolonged PSI acceptor limitation, providing a buffer to minimise ROS accumulation and damage at PSI (Noguchi, Yoshida 2008). As no malate was formed in leaves infiltrated with

MV, the primed mitochondria increased the rate of decarboxylation reactions due to an increased sink availability.

Discussion

ROS produced photosynthetically in the chloroplast's thylakoid membrane play a key role in photodamage, environmental sensing and photosynthetic regulation. Within this paradigm it is proposed that relatively stable ROS, such as H₂O₂, can be exported from the chloroplast to act directly as a retrograde signal in fine-tuning nuclear gene expression during plant acclimation to changing environments (Gollan, Aro 2020, Exposito-Rodriguez, Laissue et al. 2017), or as a trigger for cellular processes such as stomatal closure (Wang, He et al. 2016, Iwai, Ogata et al. 2019). Models of ROS signaling require that the sources of, and responses to (signaling pathway) specific ROS are well understood. Whilst chloroplast H₂O₂ signaling pathways are slowly being defined (Gollan, Aro 2020, Crisp, Ganguly et al. 2017, Bechtold, Richard et al. 2008, Rossel, Wilson et al. 2007, Dietz, Mittler et al. 2016), the endogenous sources of specific ROS in the thylakoid membrane and the environmental conditions that lead to their formation are still subject to debate. For example, the current literature supports the generation of a stable H₂O₂ pool, capable of export from the chloroplast, in multiple sites of PETC including the reduction of O₂ at PSII (Khorobrykh, Andrey 2019, Tiwari, Pospisil 2009), the PQ pool (Khorobrykh, S. A., Ivanov 2002, Mubarakshina, Ivanov 2010, Khorobrykh, Sergey A., Karonen et al. 2015), PTOX (Heyno, Gross et al. 2009), Cyt-*b₆f* complex (Baniulis, Hasan et al. 2013) and PSI (Mehler 1951, Kozuleva, Ivanov et al. 2020). This broad range of candidates complicates models of ROS signaling, resulting in debates like those between Wang et al (Wang, He et al. 2016) and Iwai et al (Iwai, Ogata et al. 2019). Whilst both agree that stomatal closure is triggered by chloroplast derived H₂O₂, the former argues in favour of reduced PQ as the source H₂O₂ and the latter concludes that it must be sourced from PSI. These discrepancies, together with our recent finding that the inhibitor DNP-INT, used historically in this field, fails to completely block re-reduction of P700⁺ (Fitzpatrick, Aro et al. 2020) prompted us to re-evaluate the ROS formation by specific PETC components of the thylakoid membrane. As the application of DNP-INT in previous studies (Borisova-Mubarakshina, M. M., Naydov et al. 2018, Khorobrykh, S. A., Ivanov 2002) likely failed to fully inhibit the PSI-Mehler reaction, artefacts may have been reported which could complicate the models of ROS signaling. By thoroughly testing the efficacy of all chosen inhibitors with P700 spectroscopy, and directly quantifying the simultaneous O₂ production and consumption reactions with MIMS, we have taken here a new approach to measuring site-specific ROS formation within the PETC, and particularly the formation of a steady pool of H₂O₂, which minimises the number of assumptions to be made during analysis.

H₂O₂ accumulation via O₂ photoreduction in isolated thylakoids occurs exclusively at PSI -
Measuring the stoichiometry of O₂ produced and consumed by isolated thylakoids during illumination enabled the calculation of an O₂ flux ratio, which was anticipated to reach 1:2

when H₂O₂ accumulated as described previously by (Allen 1977, Asada 1999, Asada 2006) (Fig. 1). As expected, we observed a 1:2 O₂ flux ratio in untreated control and MV treated thylakoid samples (Fig. 3H and 4E). Further addition of catalase to these thylakoid samples pushed the ratio back to 1:1 (Fig. 3, B and D), confirming that accumulation of H₂O₂ was responsible for the observed 1:2 O₂ flux ratio. This result fully supports the known fact that the PSI-Mehler reaction generates a stable pool of H₂O₂ (Mehler 1951). In stark contrast to O₂ flux results with control and MV treated thylakoids, the isolated thylakoids incubated with HgCl₂ and DBMIB, which we demonstrated with concomitant measurements of P700 redox kinetics to completely block all PSI function (Fig. 2, A and B), exhibited 1:1 O₂ flux ratios. These stoichiometric O₂ production and consumption experiments undoubtedly demonstrate that efficient inter-chain PETC inhibitors completely block the accumulation of H₂O₂ and, conversely, a stable pool of H₂O₂ can be acquired in isolated thylakoids only via the activity of PSI.

Separating the *formation* of H₂O₂ from its *accumulation* within the thylakoid membrane

Stable accumulation of H₂O₂ produced a 1:2 O₂ flux ratio in our experiments, being consistent to support the function of a putative direct H₂O₂ retrograde signal. In contrast, the 1:1 O₂ flux ratio that implies no stable accumulation of H₂O₂ for export, questions the involvement of thylakoid components upstream of PSI in the H₂O₂ related long distance regulatory processes. However, a significant body of previous research suggests that H₂O₂ can form upstream of PSI (the PQ pool (Khorobrykh, S. A., Ivanov 2002, Mubarakshina, Ivanov 2010, Khorobrykh, Sergey A., Karonen et al. 2015), PTOX (Heyno, Gross et al. 2009), Cyt-*b₆f* complex (Baniulis, Hasan et al. 2013)). Whilst our data seems to contradict these works, we postulate that the discrepancies merely highlight a difference in our approach, which relies on the strength of the MIMS data in separating H₂O₂ *formation* from H₂O₂ *accumulation*. Recall that illumination of control and MV treated thylakoid samples generated significant quantities of H₂O₂ (Fig. 3, A and C), yet a 1:1 O₂ flux ratio was observed in the presence of catalase (Fig. 3, B and D). In the same manner, it is plausible that H₂O₂ may form within the thylakoid membrane upstream of PSI as previously suggested (Mubarakshina, Ivanov 2010, Khorobrykh, S. A., Ivanov 2002, Khorobrykh, Sergey A., Karonen et al. 2015, Heyno, Gross et al. 2009, Baniulis, Hasan et al. 2013). Nevertheless, the MIMS data unequivocally shows that such H₂O₂ is not stable and must be rapidly broken down, analogous to the catalase treated control samples. Importantly, PSII oxygen evolution was active in the presence of DBMIB and HgCl₂ (Fig. 3, B and D), making it plausible that O₂^{•-} was formed and therefore also H₂O₂ was apparently being transiently produced in the absence of PSI. It is conceivable that such unstable H₂O₂ may be positively detected by sensitive dyes (Cathcart, Schwiers et al. 1983), spin-traps, specific sensor proteins (Villamena, Zweier 2004) or other sensitive methods of H₂O₂ detection. For this reason, the capacity of the MIMS method to discriminate between H₂O₂ that accumulated, versus H₂O₂ that briefly formed, is a functionally significant distinction from the perspective of the putative H₂O₂ retrograde signal.

Pathway(s) of O₂ photoreduction within the thylakoid membrane – MIMS measurements of thylakoid samples incubated with HgCl₂ (Fig. 3G and 4H) and both thylakoid and leaf samples incubated with DBMIB (Fig. 3F, 4G and 6D) maintained steady-state O₂ production during illumination. This PSII activity was associated with commensurate O₂ consumption resulting in a 1:1 O₂ flux ratio. Both DBMIB and HgCl₂ treated thylakoid samples exhibited similar O₂ flux rates at GL which did not increase during HL illumination in the DBMIB treated samples, a dynamic also observed in DBMIB infiltrated leaf discs (Fig. 6D and Supplemental Tab. S1,). Conversely, the O₂ flux rate doubled in response to HL in the HgCl₂ thylakoid samples (Supplemental Fig. S1, direct comparison of thylakoid samples). As the PSII contribution to O₂^{-•} formation is relatively low (Khorobrykh, Andrey 2019), the DBMIB pathway (excluding Cyt-*b₆f*) tested the maximum rate of O₂ reduction resulting from direct interaction between reduced Q_A, Q_B or the PQ pool and O₂, potentially via the theorised PQH₂/PQH⁻ O₂^{-•} formation and quenching mechanism (Khorobrykh, Sergey, Tyystjärvi 2018) and/or directly via PTOX (Cournac, Josse et al. 2000). That the rate was saturated at a low irradiance was consistent with in-vitro characterisations of the PTOX pathway (Nawrocki, Tourasse et al. 2015). In comparison, the reduction of PQ pool and Cyt-*b₆f* in HgCl₂ treated thylakoids correlated to an increased rate of both O₂ production and consumption at HL, which supports the widely held hypothesis that Cyt-*b₆f* can generate O₂^{-•} as demonstrated by (Baniulis, Hasan et al. 2013) (as HgCl₂ was toxic for leaf discs, a direct comparison between in-vitro and in-vivo HgCl₂ samples was impossible). We unexpectedly found that application of the specific PTOX inhibitors n-propyl- and octyl-gallate increased the rates of both O₂ production and consumption (Supplemental Fig. S4), confounding our attempt to examine the specific contribution of PTOX to the rate of O₂ reduction. The absence of an EPR spin-trap signal in HgCl₂ treated thylakoids supports a route of catalysed O₂ reduction within the thylakoid membrane, possibly at PTOX (Fig. 5B, green curve). These results suggest that (i) thylakoid membranes apparently support at least two separate routes of O₂ reduction upstream of PSI, (ii) both routes of O₂ reduction exhibit a lower absolute capacity than the PSI-Mehler reaction and (iii) none of the O₂ reduction pathways upstream of PSI produce a stable pool of H₂O₂ that could be exported as a retrograde signal.

A simpler model for H₂O₂ signaling - Our conclusions broadly support what is already described regarding the *formation* of ROS by photosynthetic processes in the chloroplast (Kozuleva, Ivanov et al. 2020), including our observation of multiple distinct pathways for ROS formation associated with the thylakoid membrane. We have not ruled out that O₂^{-•} and possibly H₂O₂ form upstream of PSI and can be detected by rapidly reacting dyes (Cathcart, Schwieters et al. 1983), spin-traps or specific sensor proteins (Villamena, Zweier 2004). Despite this, our results dismiss the possibility that any stable pool of H₂O₂ can *accumulate* upstream of PSI. This finding has significant consequences for models of chloroplast H₂O₂ retrograde signaling, as it implies that any H₂O₂ exported from the

chloroplast was formed at PSI. In the absence of added Fd, isolated thylakoid samples do not exhibit strong rates of O₂ reduction (Furbank, Badger 1983). This implicates the acceptor side of PSI in O₂^{-•} formation. In addition, activity of O₂ reduction was efficiently quenched by the addition of NADP⁺ (Furbank, Badger 1983), suggesting that the acceptor side capacity of PSI regulates the rate of O₂^{-•} formation. As no other potential source of H₂O₂ formation could generate a stable H₂O₂ pool within the thylakoid membrane during our experiments, the data suggest that only the PSI acceptor side contributes to H₂O₂ involved in direct H₂O₂ retrograde signaling (Gollan, Aro 2020, Exposito-Rodriguez, Laissue et al. 2017). Therefore, the export of H₂O₂ from the chloroplast specifically communicates PSI acceptor limitation to the broader cell.

Based on the available data we propose that H₂O₂ sufficiently accumulates for export from chloroplasts only under conditions where PSI acceptors are fully reduced, and the antioxidant pathways of the WWC have become overwhelmed. This is in line with the absence of catalase (which does not require reductant to function or exhibits H₂O₂ sensitivity (Mhamdi, Queval et al. 2010)) from chloroplasts, which instead rely on pathways of H₂O₂ detoxification that are sensitive to excess H₂O₂ accumulation (Kitajima, Nii et al. 2010). In this model, the peroxiredoxin, ascorbate and ascorbate peroxidase enzymes of the Mehler WWC act as a buffer, decreasing the sensitivity of any chloroplast derived H₂O₂ signal to short term and transitory stress events. Therefore any H₂O₂ retrograde signal, which is the quickest to trigger a regulatory response during HL stress (Gollan, Aro 2020), only forms once environmental conditions potentially leading to PSI damage start to dominate (Tiwari, Mamedov et al. 2016). Such a mechanism avoids nuclear responses during transient stresses, such as sun flecks or patchy cloud cover. This avoids potentially premature down-regulation of key photosynthetic processes such as light harvesting (Borisova-Mubarakshina, Maria, Ivanov et al. 2015) during otherwise low-light conditions. Whilst short term stress events can be mitigated via the enzymes of the WWC and other regulatory processes such as non-photochemical quenching (NPQ) and photosynthetic control at Cyt-*b₆f* (Shimakawa, Miyake 2018), longer term environmental shifts that lead to chronic over-reduction of the PSI acceptor pool will result in the deactivation of the WWC's antioxidant enzymes, followed by accumulation and eventual export of H₂O₂. In this model the PSI acceptor side specific H₂O₂ retrograde signal is a mechanism to convey information from chloroplasts to the nucleus when dominant environmental conditions change and the PSI acceptor side faces chronic limitation. This provides the nucleus with a mechanism to efficiently respond for acclimation to a new environment, whilst avoiding nuclear responses to short-term perturbations such as sun flecks.

Possible evidence of direct H₂O₂ triggered cooperation between chloroplast and mitochondria - In order to estimate the O₂ flux ratio associated specifically with the Mehler reaction of leaf discs, we artificially enriched the leaf disc sample atmosphere to 2% with ¹³CO₂. This enabled us to discriminate between photosynthetic assimilation of ¹³CO₂ and the efflux of respiratory ¹²CO₂ (Fig. 6). With this method we observed that leaf discs infiltrated

with MV exhibited a sharp increase in $^{12}\text{CO}_2$ efflux from mitochondrial respiration during illumination. The light dependant burst of $^{12}\text{CO}_2$ efflux was absent in leaves infiltrated with DCMU or DBMIB, excluding oxidation of the PSI acceptor side as a cause. The $^{12}\text{CO}_2$ burst was maintained in leaves infiltrated with MV + Rotenone, yet inhibited when leaves were infiltrated with both MV+catalase. These results suggest that H_2O_2 formed by MV at the acceptor side of PSI may have triggered an increase in mitochondrial respiration. This fits with a growing body of evidence that mitochondria and chloroplasts interact to optimise the photosynthetic performance. From an efficiency perspective, the organelles co-locate to improve C_3 photosynthesis by maximising the reassimilation of respired CO_2 (Busch, Sage et al. 2013). From a regulatory perspective it has long been known that mitochondria can accept excess electrons from the chloroplast's thylakoid membrane in leaves (Noguchi, Yoshida 2008) and it was recently confirmed that mitochondrial AOX expression reduces ROS damage, and was necessary for HL acclimation in green algae (Kaye, Huang et al. 2019). The term 'malate circulation' has been proposed (Zhao, Yu et al. 2020) to describe the various regulatory functions spanning both organelles, potentially including programmed cell death, that relate to the 'malate valve' (Selinski, Scheibe 2019).

To explain our observation that MV infiltration increased the CO_2 efflux measured from leaf discs during illumination in a catalase sensitive manner, we propose that H_2O_2 produced by MV was exported from the chloroplast, which then triggered upregulation or 'priming' of an oxidative pathway in surrounding mitochondria, potentially AOX (based on work with *Chlamydomonas* (Kaye, Huang et al. 2019)). However, in our experiments, where malate was not exported from the chloroplast and the mitochondria were primed by artificially produced H_2O_2 , the upregulated pathway increased the oxidative sink capacity within the mitochondria. To satisfy this increased sink, the rate of decarboxylation reactions may have been upregulated, an effect already observed in mitochondria in the absence of photosynthesis (Scarpeci, Valle 2008).

It is conceivable that the pre-incubation with MV contributed to priming this response, hence its rapid activation during illumination, although the speed of the response may be evidence that this is a critical pathway to facilitate protection of PSI during acute acceptor limitation. It precedes down-regulation of photosynthetic processes triggered by H_2O_2 retrograde signaling, which take at least half an hour (Gollan, Aro 2020), during which time the interaction may function to provide the PSI acceptor side with either NADP^+ via the malate valve (Noguchi, Yoshida 2008), or CO_2 via the upregulated carboxylation reactions observed during our measurements. The latter response could be of great significance if the stomata also close due to a H_2O_2 trigger (Iwai, Ogata et al. 2019). This explanation fits our proposed model of PSI specific H_2O_2 signaling. It suggests that H_2O_2 export from the chloroplast simultaneously signals down regulation of photosynthetic processes during prolonged acceptor limitation, and upregulation of a pathway to temporarily increase the PSI acceptor capacity via the malate valve and potentially directly via the increased availability of respired CO_2 .

Concluding remarks

We have demonstrated that a stable pool of H_2O_2 can accumulate in the thylakoid membrane only via the PSI-Mehler reaction. This explicitly excludes the involvement of other PETC components in putative chloroplastic H_2O_2 signaling pathways, with significant implications for models of H_2O_2 involvement in processes like stomatal regulation or retrograde signaling. We have confirmed the function of separate O_2 consumption pathways within the thylakoid membrane, involving the PQ pool and Cyt- b_6f , although neither demonstrated the capacity to accumulate H_2O_2 . We propose a model for chloroplastic H_2O_2 retrograde signaling based on the PSI acceptor side capacity. This enables efficient nuclear regulation in response to chronic environmental changes, whilst avoiding unnecessary responses to short term stress caused by conditions such as sun-flecks or patchy cloud cover. Finally, we have discovered potential evidence for H_2O_2 triggered cooperation between the chloroplast and mitochondria. We suggest that this may alleviate PSI acceptor side stress when the antioxidant systems of the WWC are overwhelmed and PSI is more susceptible to damage whilst nuclear regulation, activated by H_2O_2 retrograde signaling, takes effect.

Methods

Plant Material and thylakoids isolation: The *Arabidopsis thaliana* plants were grown at atmospheric CO_2 under dark/light cycle of 16/8 h at $120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Thylakoids were isolated from 6 weeks old plants using standard method as described earlier (Tiwari, Mamedov et al. 2016). All measurements were performed with freshly isolated thylakoids in measurement buffer containing: 330 mM Sorbitol, 5 mM MgCl_2 , 10 mM NaCl, 5 mM NH_4Cl , 50 mM Hepes pH 7.6, unless stated otherwise. Measurements on intact leaf or leaf discs on Dual Pam and MIMS were performed using the same plants. For infiltration of electron transfer inhibitors/modulators in leaves, detached leaves were floated at 25°C in darkness for 1 h in either H_2O (control) or $\text{H}_2\text{O} + 10 \mu\text{M}$ DBMIB or $\text{H}_2\text{O} + 10 \mu\text{M}$ DNP-INT or $\text{H}_2\text{O} + 10 \mu\text{M}$ MV.

P700 redox kinetics measurements: Detached leaves and isolated thylakoids were used for P700 redox kinetics measurements using Dual-Pam-100 (Walz, Germany). The P700 was oxidised under continuous far red (FR) light. The partial and complete re-reduction of P700 by electrons flow from PSII was achieved by shooting two short pulses of saturating actinic light pulses *i.e.* single turnover (ST) $50\mu\text{s}$ and multiple turnover (MT) 50ms , over the FR light oxidised P700 (Tiwari, Mamedov et al. 2016).

Superoxide measurement: Spin trapping for $\text{O}_2^{\cdot-}$ was performed in isolated thylakoids using Miniscope (MS5000) Electron Paramagnetic Resonance (EPR)-spectrometer equipped with variable temperature controller (TC-HO4) and Hamamatsu light source (LC8). The isolated

thylakoids equivalent to 150 $\mu\text{g ml}^{-1}$ Chl were illuminated under actinic light (150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 180 s in the presence of 5-(Diisopropoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide 2-Diisopropylphosphono-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (DIPPMPO) spin trap (50 mM) in 50 mM Hepes-NaOH (pH 7.5) with 50 μM desferol. Subsequently, the samples were centrifuged at 6500 $\times g$ for 5 min and supernatant was used for EPR measurements. The electron transport inhibitors DCMU (10 μM), DBMIB (10 μM), DNP-INT (10 μM), HgCl_2 (2 mg ml^{-1}) and MV (10 μM) were added in reaction medium when indicated prior to the measurements. The measurements were conducted at frequency 9.41 GHz, centre field 3363 G, field sweep 150 G, microwave power 3 mW and modulation frequency of 100 kHz with modulation width of 2G. The final spectra were obtained by 5 accumulations of each sample of minimum 3 to 5 biological replicates.

Gas exchange measurements in liquid samples: The MIMS liquid cuvette consisted of an in-house modified Hansatech O_2 electrode body, with the silver/platinum electrode replaced by a stainless-steel assembly supporting a Teflon membrane (Hansatech, UK) attached directly to the High Vacuum inlet of a Thermo Sentinel-PRO magnetic sector mass spectrometer (Thermo-Fisher USA), collecting masses 32 and 36 with a total cycle time of approximately 4.5 seconds. Freshly isolated thylakoid were stored on ice in darkness. For each run sufficient measurement buffer (containing: 330 mM Sorbitol, 5 mM MgCl_2 , 10 mM NaCl, 5 mM NH_4Cl , 50 mM Hepes pH 7.6) was loaded into the cuvette with the addition, via syringe (approximately 50 $\mu\text{g ml}^{-1}$ chlorophyll) to a final volume of 1000 μl . In darkness the sample was purged with N_2 to minimise background $^{16}\text{O}_2$ before a bubble of $^{18}\text{O}_2$ (99% Cambridge Isotope Laboratories Inc, UK) was loaded into the stirring liquid, bringing the concentration of the heavier isotope up to approximately 150 nmol ml^{-1} . The bubble was removed and inhibitors were injected at this moment (10 μM DCMU, 10 μM MV, 10 μM DBMIB, 10 μM DNP-INT). Samples were illuminated via halogen lamp (Dolan Jenner, USA) at low light (120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or high light (900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). At the end of each run the Chl concentration of each sample was determined in triplicate using the Porra Method (Porra, Thompson et al. 1989) in 90% MeOH to ensure accurate normalization of rates between samples. The cuvette was washed thoroughly with multiple rinses of 70% ethanol followed by MQ H_2O when changing between inhibitors to avoid cross contamination. All data was analysed and fluxes calculated with equations described in (Beckmann, Messinger et al. 2009), which includes offsets for the changing relative concentrations of $^{16}\text{O}_2$ and $^{18}\text{O}_2$, whilst any background O_2 consumption produced by thylakoid samples was normalised to zero in darkness as per (Furbank, Badger 1983).

Gas exchange measurements in leaf discs: Gas fluxes from intact leaf discs in an atmosphere enriched in two stable isotopes, $^{18}\text{O}_2$ and $^{13}\text{CO}_2$, was performed using MIMS in an in-house built 1000 μl gas-phase cuvette connected to the same mass spectrometer, collecting masses 32, 36, 44 and 45 with a cycle time of approximately 6 seconds. 14 mm diameter leaf discs cut from fully developed WT *Arabidopsis thaliana* plants floated in darkness for 4 hours in a dish of H_2O (control), $\text{H}_2\text{O} + \text{MV}$ (10 μM), $\text{H}_2\text{O} + \text{DBMIB}$ (20 μM) and

H₂O + DCMU (10 µM) at approximately 20 °C. For leaf disc samples infiltrated with catalase, SHAM and rotenone, discs were vacuum infiltrated before being allowed to dry on damp tissue in darkness for about one hour (until discs were no longer transparent). For all samples, in minimal light a 12.5 mm disc was cut from the incubated 14 mm discs, and loaded into the pre-calibrated cuvette at 25 °C. In darkness, the samples were purged with atmospheric air scrubbed of endogenous ¹²CO₂ (Soda Lime, Li-Core, USA), the cuvette was then enriched to approximately 2% ¹³CO₂ and 3% ¹⁸O₂ by volume, with the remainder of the atmosphere comprising approximately standard atmospheric concentrations. The very low ¹²CO₂ partial pressure in the beginning of measurements minimized membrane consumption of this isotope to almost zero, enabling the rate of its production in darkness (mitochondrial respiration) to be used as a basis for setting the background consumption rate of ¹⁸O₂ (mitochondrial respiration in dark, plus Mehler reaction during illumination). Rates of ¹⁶O₂ (PSII water splitting) and ¹³CO₂ (CO₂ fixation by Rubisco) were both set to zero in darkness. The enriched CO₂ atmosphere avoided photorespiration, whilst an approximately atmospheric level of O₂ was maintained to maximize the probability of observing Mehler associated O₂ reduction, with the same offsets for steady dilution of the stable isotopes used in calculations of steady state fluxes as described for liquid phase measurements. Following initiation of data acquisition, samples experienced three minutes of darkness, five minutes of growth light (120 µmol photons m⁻² s⁻¹), five minutes of high light (900 µmol(photons) m⁻² s⁻¹) and then four minutes of darkness. Rates were calculated according to equations described in (Beckmann, Messinger et al. 2009).

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