The multiple routes of photosynthetic electron transfer in Chlamydomonas reinhardtii

Dany Croteau, Jean Alric, Benjamin Bailleul

Chapter 18: The multiple routes of photosynthetic electron transfer in *Chlamydomonas reinhardtii*

Dany Croteau[1], Jean Alric[2], Benjamin Bailleul[1]

[1] Institut de Biologie Physico-Chimique, Laboratory of Chloroplast Biology and Light Sensing in Microalgae, UMR 7141, Centre National de la Recherche Scientifique (CNRS), Sorbonne université, F-75005 Paris, France

[2] Aix Marseille Univ, CEA, CNRS, BIAM, UMR 7265, Photosynthesis and Environment, F-13108 Saint Paul-Lez-Durance, France

Non-print items - Abstract

Like all organisms performing oxygenic photosynthesis, *Chlamydomonas* captures light energy in two photochemical steps to drive linear electron flow from water to NADPH and to produce ATP. However, this process alone is not sufficient to drive CO_2 fixation in the Calvin-Benson-Bassham cycle and to respond to environmental and metabolic constraints, e.g. light availability or metabolic needs in term of ATP and NADPH. A complex network of alternative electron flows, which comprise the Cyclic Electron Flow around PSI or the various water-to-water cycles utilising O_2 as alternative electron acceptor, provide an additional degree of freedom to face this challenge. The present chapter presents the various alternative routes of photosynthetic electron transfer in *Chlamydomonas*, describes how they coordinate to optimize photosynthesis and gives a retrospective on the early physiology work on those alternative electron flows across model photosynthetic organisms.

Non-print items – Keywords: Chlamydomonas, Alternative electron flows, photosynthesis, Mehler, flavodiiron proteins, PTOX, Cyclic Electron flow.

Abbreviations:

PETC: Photosynthetic electron transport chain

PSI/PSII: Photosystem I / II

cyt. b_6f: cytochrome b_6f complex

LEF: linear electron flow

PQ/PQH_2: plastoquinone/plastoquinol

PC: plastocyanin

FNR: $Fd:NADP^+$ reductase

$NADP^+$/NADPH: dinucleotide phosphate

$\Delta\mu_{H+}$: electrochemical proton gradient

ΔpH: proton concentration gradient

$\Delta\psi$: electric potential

CBB: Calvin-Benson-Bassham

NPQ: Non-Photochemical Quenching of chlorophyll fluorescence

qT: state transition

qE: energy-dependent quenching

AEF: alternative electron flows

CEF: Cyclic Electron Flow

WWC: Water-to-Water Cycle

ROS: Reactive Oxygen Species

LHCII: Light Harvesting Complex II

PTOX: plastid terminal oxidase

Fd: ferredoxin

FQR: Fd-PQ oxidoreductase

PGR5: *proton gradient regulation 5*

PGRL1: PGR5-like 1

NDH: nucleotide dehydrogenase

WT: wildtype

FLV: Flavodiiron protein

AsA: Ascorbate

MDA: monodehydroascorbate

PhQ: phylloquinones

CCM: carbon concentration mechanisms

GlcDH: glycolate dehydrogenase

Introduction

Like all organisms performing oxygenic photosynthesis, *Chlamydomonas* captures light energy in two photochemical steps to drive the photosynthetic electron transport chain (PETC) in the thylakoid membrane. Electron transport involves the action of three major molecular complexes embedded in the thylakoid lipid phase: the photosystems (PS) I and II (Volume 2, Chapter 15) and the cytochrome b_6f complex (cyt. b_6f, Chapter 16). In the main linear electron flow (LEF) pathway (Fig 1), the primary electron donor, water, is oxidized at the PSII reaction center. Both PS work in series (Hill & Bendall, 1960) transferring electrons along redox cascades. Between photosystems, electrons transit through the cyt. b_6f via two mobile carriers, the hydrophobic and membrane soluble plastoquinone (PQ)-pool connecting PSII to cyt. b_6f and the water-soluble plastocyanin (PC) shuttle connecting cyt. b_6f to PSI in the thylakoid lumen. At the PSI acceptor side, Fd:$NADP^+$ reductase (FNR) accepts electrons from Fd to reduce dinucleotide phosphate ($NADP^+$) into NADPH. The LEF allows water splitting on one side, production of reducing power on the other side, and the generation of an electrochemical proton gradient ($\Delta\mu_{H+}$) across the membrane. Proton accumulation in the lumen is coupled to LEF through water splitting at PSII, and through the Q-cycle at the cyt. b_6f (Joliot & Joliot, 2006). The proton-motive force $\Delta\mu_{H+}$ comprises two energetic components, a proton concentration gradient (ΔpH) and an electric potential ($\Delta\psi$) both driving CF_1F_O-ATP synthase catalytic rotation (Mitchell, 1966; Kramer *et al.*, 2003) (Volume 2, Chapter 16). The intermediate molecules NADPH and ATP are the two energy

sources fueling the Calvin-Benson-Bassham (CBB) cycle (Volume 2, Chapters 7 and 8).

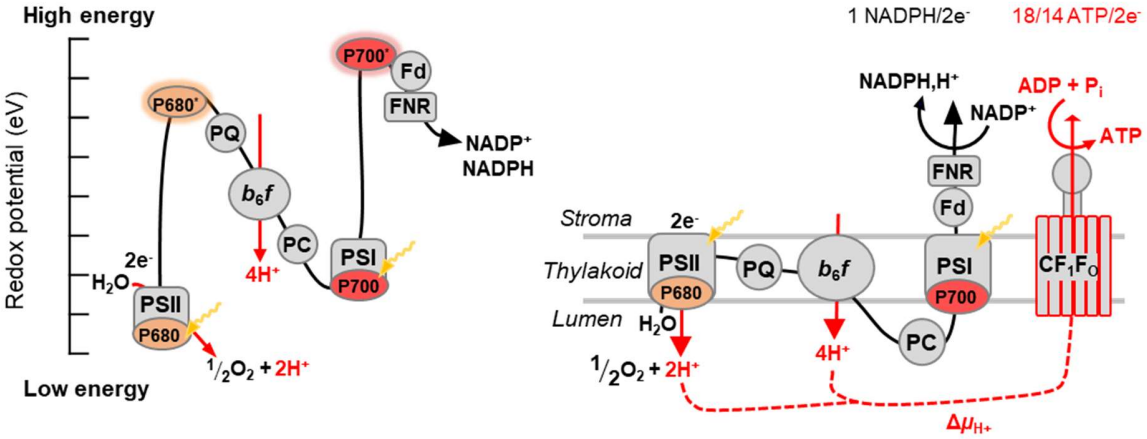


Figure 1: the photosynthetic Linear Electron Flow (LEF). A. LEF in the Z scheme of photosynthesis. B. Representation of the LEF in the thylakoid membranes. Abbreviations: PSII, Photosystem II; PSI, Photosystem I; b_6f, cytochrome b_6f; CF_1F_O, CF_1F_O ATPase; PC, plastocyanin; PQ, Plastoquinol/Plastoquinone; Fd: ferredoxin; FNR: Ferredoxin/NADP Reductase; $\Delta\mu_{H^+}$, electrochemical proton gradient.

A description of the photosynthetic electron transfer chain restricted to LEF would give an overly simplistic view of the photochemical phase of photosynthesis. LEF produces a fixed ratio of ATP and NADPH, and in a context were the light input and the metabolic demand in ATP and NADPH are constantly changing, photosynthesis gets the best of LEF only by allowing it to work with some "degrees of freedom". One of them is the biological regulation of the light utilization by the two photosystems, through the non-photochemical quenching (NPQ) at the PSII (Volume 2, Chapter 25) or state transition (qT) (Volume 2, Chapter 24) which is a process particularly important in *Chlamydomonas*. Another main mode of regulation of the photochemical phase of photosynthesis relates to the flexibility of photosynthetic electron flows. In all organisms performing oxygenic photosynthesis, LEF components (PSI, PSII, cyt b_6f) are always involved in

95 a complex network of alternative electron flows (AEFs) which divert electron transport from
96 NADPH production or assimilation of CO_2. The present chapter focuses on those AEFs and is
97 structured as follows; in Section 1, we first explain the input and output constraints on LEF
98 operation (light availability and metabolic needs in term of ATP and NADPH) and how AEFs
99 provide an additional degree of freedom to face this challenge. Then we present the various AEFs
100 in Section 2, from the first experimental evidence to recent works more specific to
101 *Chlamydomonas*. We will especially focus on the two main AEFs modes (schematized in Fig 2
102 and 3), the Cyclic Electron Flow (CEF) around PSI, where electrons at the acceptor side of PSI
103 are reinjected upstream the cyt. b_6f, and the various water-to-water cycles (WWC) (or pseudo-
104 cyclic electron fluxes), which utilise O_2 as alternative electron acceptor instead of CO_2 (Curien *et*
105 *al.*, 2016). In Section 3, we discuss how the various AEFs coordinate to optimize photosynthesis
106 in response to changes in environmental conditions. Finally, in relation to the adaptive role of
107 AEFs in green algae, this review ends with a retrospective on the early physiology work on AEFs
108 across model photosynthetic organisms.

109

110 ## 1. AEFs provide flexibility to the Linear Electron Flow

111 The operation of LEF, which uses light as a substrate and synthetizes ATP and NADPH as
112 end-products, is constrained at these two extremities. First, when light fluctuates, light absorption
113 must be adjusted to the electron transport capacity; and second, the ATP/NADPH ratio must adjust
114 to the metabolic needs of the moment. All AEFs share some common features: they play a role of
115 valves, offering an outlet for an additional activity of one photosystem or both. By doing so, they
116 also allow the pumping of extra protons into the lumen, thereby driving an extra $\Delta\mu_{H+}$ and ATP

117 production. In this first section we will describe first how those common features of AEFs help
118 responding to light and metabolic constraints. Only in the Section 2 which describes AEFs one by
119 one, will their differences and specificities be pointed out, e.g., in terms of photon budget for
120 proton pumping and ATP production.

121 Exposure to excess light can lead to over-reduction of PS acceptor-side electron carriers
122 (iron-sulfur centers, Fd and NADPH at PSI; Q_A, Q_B and the PQ pool at PSII), augmenting the
123 chances for excited chlorophyll *a* to transition into a highly unstable triplet state reacting with O_2.
124 The latter can result in the production of Reactive Oxygen Species (ROS) and photooxidative
125 damage, including photoinhibition of the two PSs (Volume 2, Chapter 25). At PSII, the induction
126 of a series of mechanisms, collectively referred to as NPQ allow to diminish the light pressure on
127 PSII (Volume 2, Chapter 25). In *Chlamydomonas*, the major component of NPQ is the energy-
128 dependent quenching (qE) which dissipates excess light energy as harmless heat, and prevents
129 photoinhibition of both PS (Roach *et al.*, 2020). Another NPQ process particularly important in
130 *Chlamydomonas* is state transition (qT) where a reversible kinase-dependent migration of light
131 harvesting antennae LHCII from one photosystem to the other modulates PSII and PSI light
132 harvesting and rebalances energy fluxes (Volume 2, Chapter 24). The two processes are regulated
133 differently since qE depends on the luminal pH whereas the activity of the kinase involved in the
134 qT is regulated by the redox state of the PQ in the Q_o site of the cyt. b_6f (Wollman, 2001; Dumas
135 et al., 2017). When considering the necessary adjustment of the light absorption to the capacity of
136 electron transfer, NPQ manages the downregulation of the former where AEFs allow the
137 upregulation of the later. Therefore, by providing an alternative route for excess electrons, AEFs
138 play the role of redox exhaust valves and protect PSs from photoinhibition.

In addition to the response to light changes, the PETC also faces a constraint related to the stoichiometry of NADPH and ATP. The LEF produces ATP from H^+ accumulated in the lumen and NADPH from e^- transfer along the PETC. Because of the H^+/e^- coupling, ATP/NADPH production is fixed in LEF. The water oxidation at the PSII level produces one proton in the lumen per electron transferred. At the level of the cyt. b_6f, 2 protons are pumped per electron transferred along the chain towards PC, thanks to the Q-cycle (Volume 2, Chapter 17). PQH_2, which acquired two protons from the stroma when reduced at the Q_B site, is oxidized to PQ at the luminal facing Q_o site of cyt. b_6f, where the two transported electrons take bifurcated pathways but both protons are released in the lumen. One electron follows the high potential chain of the Rieske iron-sulphur protein and cyt. *f* and attains PC. The second electron follows the low potential chain via haem b_L and haem b_H. In Mitchell's original proposal (1975), this electron in the low potential chain was used to reduce PQ to PQH_2 on the stromal side of the membrane via an electron coming from PSII. However, based on works on the respiratory cyt. bc_1, a modified Q-cycle was proposed where the *b* haems can act as an electron reservoir and that PQH_2 is solely fully regenerated after two cycles (Zhang *et al.*, 1998; Crofts *et al.*, 2003; Cramer *et al.*, 2011). The modified Q-cycle is largely accepted nowadays, which is constitutively at place and transfers two protons per electron (Sacksteder et al. 2000; but see section Cyclic Electron Flow). Altogether, the LEF produces 3 H^+ per electron but, from a metabolic perspective, what matters is the relative amount of reducing power and ATP produced by the PETC, often reflected by the ATP/NADPH ratio. Since two electrons are required to reduce $NADP^+$, this leads to a value of 6 protons per NADPH. The question mark lies in the proton requirement per ATP synthesized, which can vary between species depending on the stoichiometry of the subunits forming the membrane intrinsic *c*-ring of the CF_1F_O-ATP synthase (Petersen *et al.*, 2012; Turina *et al.*, 2016). For most authors, each proton

crossing the CF_1F_O-ATP synthase induces a one incremental step rotation of the *c*-ring on the F_O sector transduced via a central stalk to catalytic conformational changes of the $\alpha_3\beta_3$ subunits on the F_1 sector. Although it remains uncertain whether the work accomplished by each translocated proton is homogeneous (Turina *et al.*, 2016), the yield of 3 ATP molecules synthesized/hydrolysed per CF_1F_O 360º rotation is well established (Adachi *et al.*, 2007). No structural information of the CF_1F_O-ATP synthase allows to give a definitive stoichiometry in *Chlamydomonas*, but the 14 *c*-subunits of the ATP synthase rotor in spinach is usually used as a reference for eukaryotic photosynthetic organisms (Seelert *et al.*, 2000; Hahn *et al.*, 2018). Then 6 protons released in the lumen yield ~1.28 ATP (6*3/14) and the 3 H^+/e^- coupling leads to 1.28 ATP per NADPH. This ratio is below the 3/2 requirement of the CBB cycle (Allen, 2002). Consequently, without additional flexibility, LEF from water to carbon assimilation is condemned to accumulating NADPH until the photosynthetic activity is fully arrested. AEFs permit an additional activity of the photosynthetic complexes involved in proton pumping, PSII and/or the cyt. b_6f. From this point of view, AEFs can be seen as degrees of flexibility for the electron transfer, allowing for proton pumping without net production of NADPH. This, in turn, generates extra ATP and respond to the mismatch between the ATP/NADPH produced by the PETC and the ATP/NADPH requirement for carbon assimilation. Many reports aimed at calculating the required activity of AEFs to adjust the ATP/NADPH ratio of the CBB cycle (Allen, 2002, Amthor, 2010, Alric et al., 2010) and conclude that AEFs should accumulate one additional proton in the lumen per NADPH produced by the LEF (6 +1 = 7 protons per NADPH would produce 7*3/14 = 1.5 ATP).

However, it would be naive to see AEFs solely as a balance for the shortcomings of the 14 *c*-subunits of the CF_1F_O ATP synthase. In the stroma, the reducing power in the form of reduced ferredoxin or NADPH not only drives the CBB cycle but also stromal enzymes involved in

essential metabolic pathways like nutrients assimilation (Volume 2, Chapter 4) or amino acid biosynthesis (Volume 2, Chapter 6). Moreover, the substrate availability for the PETC (light) and for the CBB cycle (inorganic carbon) can largely and suddenly vary, limiting the general relevance of such a static view. In low CO_2 conditions, carbon concentration mechanisms (CCM) are induced to gather high CO_2 density around Rubisco confined in pyrenoids (reviewed in *Chlamydomonas* in (Wang *et al.*, 2015), see also Volume 2, Chapter 7). Due to the ATP demand of CCMs, the ATP/NADPH ratio for carbon assimilation is further influenced by CO_2 availability. The network of AEFs in organisms performing oxygenic photosynthesis, including *Chlamydomonas*, should be seen as a dynamic flexibility to abiotic conditions and metabolic needs, by fulfilling three major roles: (i) AEFs can dynamically optimize the ATP/NADPH ratio produced by the PETC to the metabolic needs, and thereby avoid the ATP/NADPH stoichiometric cul-de-sac intrinsic to the sole LEF. (ii) They offer electron valves in conditions where light absorption exceeds the capacity of the sole LEF, preventing the over-reduction of the PETC and risks of photoinhibition. (iii) Finally, by generating additional $\Delta\mu H^+$, AEFs participate to the pH-mediated regulation of the PETC under saturating light conditions.

The pH control of the PETC comprises the regulation of NPQ (Volume 2, Chapter 15) and the so-called "photosynthetic control". In conditions of electron transfer limitation downstream of PSI (e.g., low temperature or low CO_2), PSI can be photoinhibited (Chaux et al., 2015). The "photosynthetic control" (Volume 2, Chapter 17) which occurs when a low luminal pH slows down PQH_2 oxidation at the Q_o site of the cyt. b_6f (Bendall, 1982), prevents this harmful situation by moving the limiting step of the PETC limiting upstream of PSI (Sukenik *et al.*, 1987). The redox regulation of the CF_1F_O-ATP synthase, which is not covered in this chapter (but see Volume 2, Chapter 17), also plays a crucial role in the modulation of the ΔpH (Kramer *et al.*, 1990). The γ-

subunit of the CF_1F_O-ATP synthase contains a cysteine couple permitting a disulfide formation under light which decreases the $\Delta\mu_{H+}$ required for CF_1F_O-ATP synthase rotation (Junesch & Gräber, 1987). This mechanism is conserved and restricted to the green lineage, including *Chlamydomonas* (Buchert *et al*, 2017; Buchert *et al.*, 2021).

2. Diversity of AEFs in Chlamydomonas

With the definition used in this chapter for AEFs, i.e. electron routes involving photosynthetic complexes (PSII, PSI, cyt b_6f) without resulting in CO_2 fixation, the diversity of AEFs in *Chlamydomonas* is very large. Some of them are already covered in other chapters: one can consider that the use of photosynthetically reduced ferredoxin or NADPH for nutrient assimilation, e.g., nitrogen or sulfur metabolism (Volume 2, Chapter 4) or for amino acid biosynthesis (Volume 2, Chapter 6) enter that category. This is also the case of the coupling between the PETC and the hydrogenase which uses reduced ferredoxins as substrate, in anaerobic conditions (Volume 2, Chapter 9 and 10). In a way, the recombination events in PSs meet the definition strictly speaking but are covered already in the chapter dedicated to photosystems (Volume 2, Chapter 16) and will not be considered here. Finally, the chlororespiration pathway is a minimal respiratory chain in the thylakoid and cannot be considered an AEF but its components are involved in AEFs. This section will especially cover PSI CEF and the various WWCs.

2.1 Chlororespiratory pathway

A minimal respiratory pathway exists in the thylakoids of most organisms performing oxygenic photosynthesis. Bennoun (1982) first reported that PQH_2 could be oxidised in darkness by a plastid terminal oxidase (PTOX). PTOX, together with a NAD(P)H plastoquinone oxidoreductase, forms a chlororespiratory transport chain using electrons from NAD(P)H to reduce O_2 into H_2O (see

review on PTOX (Nawrocki *et al.*, 2015)). In plants, PTOX was initially investigated to decipher its role in chloroplast development and carotenoids biosynthesis (Wu et al., 1999), presumably by regenerating (oxidized) PQ in darkness, which acts as an electron acceptor to activate the phytoene desaturase upstream of the carotenoids' synthesis pathway (Carol & Kuntz, 2001; Bennoun, 2002). Two PTOX enzymes are expressed by *Chlamydomonas*, PTOX2 being the main oxidase with a rate ~10 fold faster than PTOX1 (4.5 vs. 0.4 e^- PS^- s^{-1}) (Houille-Vernes *et al.*, 2011). *Chlamydomonas* phytoene desaturase mutants show similar phenotypes to plants mutants with regards to carotenoids synthesis (McCarthy *et al.*, 2004; Couso *et al.*, 2011). However, demonstrations of PTOX's involvement in this pathway remain inconclusive, notably because *ptox2 Chlamydomonas* mutants (see *Chlamydomonas* AEF mutants strains in Table 1) do not show changes in carotenoids content (Houille-Vernes *et al.*, 2011). This function could be undertaken by PTOX1 (Li *et al.*, 2020) as suggested in the green algae *Haematococcus pluvialis* (Wang *et al.*, 2009); however, two observations make the role of PTOX in carotenoid synthesis of green algae unclear. Not only the O_2 uptake by PTOX during the dark segment of *Chlamydomonas'* photoperiod is almost null (Strenkert *et al.*, 2019) but, in contrast to plants, most carotenoids seem to be synthesized under light in both *Chlamydomonas* (Janeiro & Barrnett, 1982) and *H. pluvialis* (Wang *et al.*, 2009).

In plants, the chlororespiratory pathway also involves a chloroplast nucleotide dehydrogenase (NDH) complex, called NDH-1. NDH-1 is closely related to mitochondrial respiration complex I (Sazanov *et al.*, 1998), contains 11 subunits with multiple transmembrane domains and reduces quinones with electrons from reduced Fd. *Chlamydomonas* and other green algae possess instead the NAD(P)H dehydrogenase 2 (NDA2) (Jans *et al.*, 2008), which exhibit similar function as NDH-1 in cyanobacteria and higher plants. NDA2 also utilizes electrons from NAD(P)H to non-

253 photochemically reduce the PQ-pool, thanks to a non-covalently bound flavin mononucleotide co-
254 factor (Jans *et al.*, 2008; Desplats *et al.*, 2009). But it also shows important disparities with NDH-
255 1 (reviewed in (Peltier *et al.*, 2016)): it is a monomeric flavoenzyme membrane-bound on the
256 stromal side of the thylakoid and it uses NAD(P)H as a substrate (Jans *et al.*, 2008). Moreover,
257 NDA2 is non-electrogenic (Melo *et al.*, 2004), opposingly to NDH-1 (Strand *et al.*, 2017).
258 Although *Chlamydomonas'* NDA2 can utilize NADPH, it favors NADH as a substrate (Desplats
259 *et al.*, 2009). Interestingly *Chlamydomonas'* plastid genome encodes for a transhydrogenase which
260 could convert NADPH to NADH in the stroma (Mus *et al.*, 2007).

261 Although chlororespiration was initially proposed to generate an electrochemical proton gradient
262 in *Chlamydomonas* (Bennoun, 2002), this model was later disproven. The electrochemical proton
263 gradient in the dark stems from the hydrolysis by the CF_1F_O-ATPase of ATP from mitochondrial
264 origin or in the absence of ATP hydrolysis by an ATP dependent ionic pump (Bennoun, 1994).
265 Because of the absence of electrogenicity of NDA2 and the position of PTOX on the stromal side
266 of the thylakoid, the chlororespiratory pathway is now considered non-electrogenic in
267 *Chlamydomonas*. Strictly speaking, chlororespiration cannot be considered a photosynthetic AEF
268 because it does not involve photochemical event. However, the two enzymes NDA2 and PTOX
269 are independently involved in CEF and WWC, respectively (see 2.2 and 2.3).

270 **2.2 Cyclic electron flow around PSI**

271 Like in other organisms, *Chlamydomonas* shows a fast ($t_{1/2}$ ~100ms) reduction of P_{700+} at
272 the offset of an illumination, in the presence of DCMU (Maxwell & Biggins, 1976), but this phase
273 is largely slowed-down in the presence of a cyt. b_6f inhibitor (Canaani *et al.*, 1989). This was seen
274 as evidence for an electron transfer from the PSI acceptors in the stroma towards P_{700+} via cyt. b_6f.
275 If the existence of a CEF around PSI is well accepted in *Chlamydomonas* (Finazzi *et al.,* 1999,

Iwai *et al.,* 2010, Alric et al., 2010) the question of the electron transfer pathways from PSI acceptors to the cyt. b_6f, the identity of the molecular players and the maximal rate of CEF have given rise to intense debate in the last decades (recently reviewed in (Nawrocki *et al.,* 2019a)).

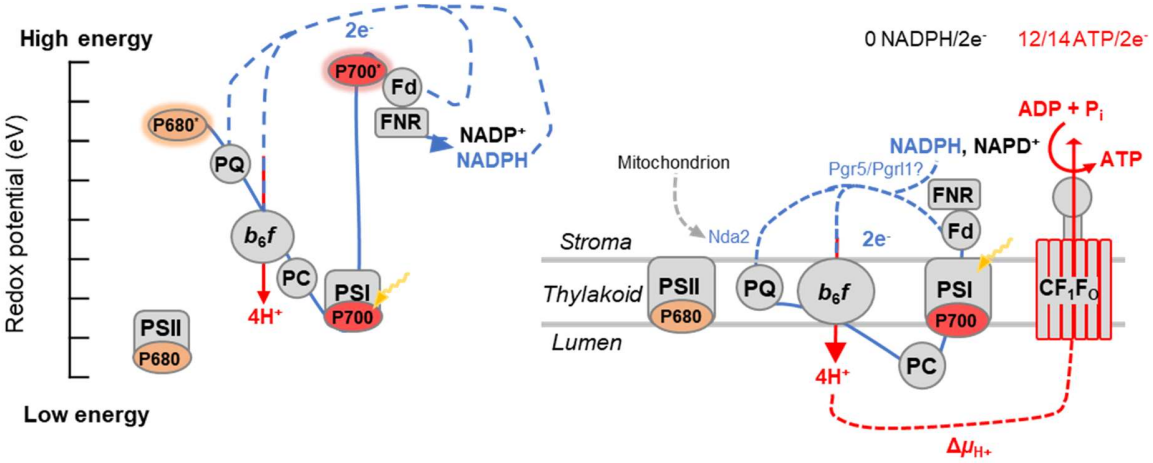


Figure 2: the Cyclic Electron Flow (CEF) around Photosystem I. A. CEF in the Z scheme of photosynthesis. B. Representation of the different routes of the CEF in the thylakoid membranes. Abbreviations: PSII, Photosystem II; PSI, Photosystem I; b_6f, cytochrome b_6f; CF_1F_O, CF_1F_O ATPase; PC, plastocyanin; PQ, Plastoquinol/Plastoquinone; Fd: ferredoxin; FNR: Ferredoxin/NADP Reductase; $\Delta\mu_H^+$, electrochemical proton gradient.

The possible routes for CEF in Chlamydomonas

Like in plants (Shikanai, 2007), two major CEF routes have been described in *Chlamydomonas* (Figure 2). The "major one" corresponds to the original pathway postulated by Arnon and co-workers (Arnon *et al.*, 1954) following the observation of light-dependent ATP phosphorylation in isolated chloroplasts, mediated by the stromal electron carrier ferredoxin (Fd) A membrane-bound Fd-PQ oxidoreductase (FQR) was proposed to reinject electrons from the PSI

292 acceptor side to the inter-system ETC (Moss & Bendall, 1984; Bendall & Manasse, 1995). This
293 pathway was serendipitously "rediscovered" almost 50 years later in a screening for *Arabidopsis*
294 qE-defective mutants, lacking a small, stromal-soluble protein named PGR5 (for *proton gradient*
295 *regulation 5*) (see Table 1) (Munekage *et al.*, 2002). Shortly after, the molecular partner of PGR5,
296 PGRL1 (PGR5-like), was discovered, which is an integral thylakoid protein with both termini
297 stroma-exposed and six redox-active cysteine residues (DalCorso *et al.*, 2008). In *Arabidopsis*,
298 Hertle and co-authors (2013) (but see also (Szymańska *et al.*, 2011)) reported *in vitro* FQR activity
299 from PGRL1 (albeit on a non-physiological quinone species) which they attributed to its redox-
300 active cysteine residues presenting a binding site for a Fe-containing cofactor and triggered by
301 PGRL1 heterodimerization with PGR5 (however see (Strand *et al.*, 2016) for critical discussion
302 on this hypothesis). While both PGR5 and PGRL1 have been shown to modulate *Chlamydomonas*
303 CEF regulation (Alric, 2014; Johnson *et al.*, 2014; Jokel *et al.*, 2018; Buchert *et al.*, 2020), their
304 direct involvement in electron recycling from PSI acceptor to the PQ-pool remains debated
305 (Nawrocki *et al.*, 2019a). Although this PGR5/PGRL1 pathway was sensitive to inhibition by
306 antimycin-A (a mitochondrial Complex III inhibitor) in plants, it is not the case in *Chlamydomonas*
307 (Iwai *et al.,* 2010, Antal *et al.,* 2013).

308 The second CEF pathway was first described in *Synechocystis* mutated in the membrane-
309 bound, nucleotide dehydrogenase (NDH) gene (Ogawa, 1991; Mi *et al.*, 1995). In the mutants the
310 PQ-pool remained oxidized after illumination in the presence of PSII inhibitor DCMU, as opposed
311 to the wildtype where PQ re-reduction was attributed to NDH-1 (Mi *et al.*, 1995). In
312 *Chlamydomonas* and other green algae, the secondary CEF pathway involves a different NAD(P)H
313 dehydrogenase, NDA2 (Jans *et al.*, 2008), already described in the chlororespiratory section (see
314 2.1). In CEF, NDA2 utilize electrons from NAD(P)H to non-photochemically reduce the PQ-pool

315 and *nda2* mutants show a shorter post-illumination fluorescence rise than wild-type because
316 NAD(P)H can not be mobilized to reduce PQ (Jans *et al.*, 2008).

317 The light sensitivity of *Arabidopsis* mutants lacking both NDH and PGR5/PGRL1
318 pathways confirmed the essential role of CEF in photosynthesis (Munekage *et al.*, 2004). It also
319 suggested that these two routes, functionally redundant, represented the only possibilities for CEF
320 in plants. However, emerging structural information on cyt. b_6f (Stroebel *et al.*, 2003; Malone *et*
321 *al.*, 2019) have stimulated the emergence of a third potential pathway for CEF in *Chlamydomonas.*
322 In this model, the CEF cycle could be completed via the direct reinjection from a stromal electron
323 donor to the cyt. b_6f following the classical Q-cycle originally proposed by Mitchell (1975). This
324 model, first proposed by Chain (1982) and reconsidered more recently on the basis of kinetic
325 considerations (Joliot & Johnson, 2011; Nawrocki *et al.*, 2019a; Buchert *et al.*, 2020), does not
326 involve the lateral diffusion of PQ in the thylakoid membrane. It supposes that the reduction of the
327 quinone at the Q_i site is bifurcated, one electron coming from a stromal reductant, the other from
328 the low-potential chain.

329 ***The maximal rate of CEF in Chlamydomonas***

330 To evaluate the physiological importance of the CEF around PSI, the value of the maximal rate of
331 CEF, relatively to the maximal rate of the LEF, has been a long-lasting question. In
332 *Chlamydomonas*, the maximal rate of LEF is usually in the 100-150 electron per second per
333 photosystem (Maxwell and Biggins 1976, Alric, 2010; Nawrocki *et al.*, 2019b). In the presence of
334 DCMU, the rate of P_{700}^+ re-reduction at the offset of a saturating light was used as a direct measure
335 of the rate of CEF and provided typical values of 5-15 electrons per second per PSI in oxic
336 conditions (Maxwell & Biggins, 1976, Alric *et al.,* 2010; Takahashi *et al*., 2013). Such a rate of
337 CEF would be insufficient to supply the LEF with the additional ATP required for carbon

assimilation, alone. However, the rate of CEF in the presence of DCMU is not always a valid estimation of the rate of CEF in physiological conditions. If LEF and CEF are in competition for a common substrate, oxidized PQ or reduced ferredoxin, inhibition of LEF would increase CEF (Fan et al, 2016). In contrast, CEF can be limited by the lack of reduced ferredoxin / NADPH (Alric *et al.*, 2010; Lucker & Kramer, 2013). This latter hypothesis is supported by higher rates of CEF measured in anoxic conditions, where the reducing power generated by glycolysis in the chloroplast maintain reducing pressure on the PETC. In these conditions (DCMU and anoxia), rates as high as 60 electrons per second per PSI were measured (Alric *et al.,* 2010), which were further corroborated during the first seconds following a dark-to-light transition in the absence of DCMU (Nawrocki *et al*, 2019b).

With this value in mind, it becomes possible to evaluate the relevance of the NDA2 and PGR5/PGRL1 pathways for CEF on a kinetic basis. The slow rate of NDA2 under aerobic conditions (2-4 $e^- PSI^{-1} s^{-1}$ (Houille-Vernes *et al.*, 2011) suggested that its role may be restricted to chlororespiration (Nawrocki *et al.*, 2015). However, during N-deprivation inhibiting the CBB and leading to the accumulation of stromal reductants, a doubling of the rate of P_{700}^+ re-reduction, dependent upon NDA2 but not upon PGR5/PGRL1, was reported (Saroussi *et al.*, 2016). This discrepancy could stem from a different redox state of the stromal reductants in the two experiments: the rate of NDA2 would be limited by the availability of NAD(P)H in oxic conditions. In *Chlamydomonas*, PGRL1 mutants were shown to exhibit transiently the same maximal CEF rate as wildtype (Nawrocki *et al.*, 2019b). This was also the case in PGR5 mutants of *Arabidopsis* where the CEF maximal rate was not affected compared to the wild type (Nandha *et al.*, 2007). In both cases, it was suggested that PGR5 and PGRL1 could play a regulatory role in CEF, possibly via the redox poising of the chloroplast, instead of playing a direct FQR function.

The supposed low rate of NDA2, together with the unchanged maximal rate of CEF in *pgrl1* mutants, were taken as clues for the existence of another CEF pathway involving the reinjection of electrons in the Q_i site of the cyt. b_6f and able to take in charge the high rates of CEF measured in *Chlamydomonas*. It is to note, however, that a compensation by NDA2 in *Chlamydomonas pgrl1* mutants can not be fully excluded yet. A NDA2/PGRL1 double mutant in *Chlamydomonas* would help testing the redundancy of NDA2 and PGR5/PGRL1 pathways and/or the hypothesis that none of them represent the major route of CEF.

A classical (Mitchellian) Q-cycle involved in CEF?

Independently from the kinetics analysis discussed above, a regain of interest for a classical Q-cycle mediated CEF model comes from crystallography and structural analysis of the differences between cyt. b_6f and cytochrome bc_1 (see also Volume 2, Chapter 11 and Chapter 17). Notably cyt. b_6f possesses three unique prosthetic groups per monomer: one chlorophyll *a*, one β-carotene and one special haem c_i (Stroebel *et al.*, 2003) which could be involved in direct electron transfer from reduced ferredoxin to the quinone in the Q_i site (Stroebel *et al.*, 2003; Malone *et al.*, 2019). Haem c_i is unique by its lack of coordination of the haem iron (no amino acid axial ligand) and by being covalently bound in the vicinity of the high potential b_H at the Q_i site (Stroebel *et al.*, 2003; Alric *et al.*, 2005; Yamashita *et al.*, 2007) yielding strong magnetic dipolar coupling between the two haems (Baymann *et al.*, 2007). Interestingly, FNR is known to interact with cyt. b_6f stromal surface (Clark *et al.*, 1984; Zhang *et al.*, 2001; Mosebach *et al.*, 2017) and FNR has been proposed to tether reduced Fd in proximity to the two high potential haems enabling PQ reduction which would assign the FQR function to cyt. b_6f (Joliot & Johnson, 2011; Goss & Hanke, 2014; Nawrocki *et al.*, 2019a). New structural insights on cyt. b_6f so far seems to support this model (Malone *et al.*, 2019) which resembles the Mitchellian Q cycle model with one electron coming

384 from the stroma. Especially, the propionate of haem c_i can switch conformation upon PQ binding
385 to the Q_i pocket, which could reflect the previously documented decrease in c_i redox potential in
386 dark-adapted algae (reducing conditions) (Alric *et al.*, 2005) and give a mechanistic basis for
387 electron channelling from the FNR:Fd complex to PQ mediated by haem c_i (Malone *et al.*, 2019).
388 Highlighting the homodimer structure of cyt. b_6f, its central cavity and the edge-to-edge distance
389 allowing electron transfer between opposing haems b_L (Lanciano *et al.*, 2013), Nawrocki et al.
390 (2019a) proposed that oxidized PQ in the Q_o site of one monomer could be shuttled to the Q_i site
391 of the other monomer. In this model, CEF and LEF would not compete for a common pool of
392 oxidized PQ; instead PSII would reduce the PQ pool freely diffusing in the thylakoid membrane,
393 whereas the CEF would inject an electron in a PQ located in the cyt. b_6f cavity, allowing high
394 CEF even under conditions where the PQ pool is highly reduced by LEF. Interestingly, a natively
395 bound PQ molecule was observed by cryo-electron microscopy between haems c_i and b_H of
396 opposing cyt. b_6f monomers, which could represent a glimpse at this putative trans-monomer
397 shuttle (Malone *et al.*, 2019). By measuring redox kinetics associated to the different haems of cyt.
398 b_6f, Buchert et al. (2020) proposed that the switch between the modified Q-cycle associated to
399 LEF and a classical Q-cycle driving CEF requires PGR5, explaining the different reports of CEF
400 phenotypes in *pgr5* mutants (Munekage *et al.*, 2002; Alric, 2014; Johnson *et al.*, 2014; Godaux *et*
401 *al.*, 2015).

402 ***Influence of Supercomplexes and State transitions on CEF***

403 Various early studies (Canaani *et al.*, 1989; Herbert *et al.*, 1990; Finazzi *et al.*, 2002) indicated a
404 relationship between state transitions, which regulates the attachment of LHCII to PSII (state 1) or
405 PSI (state 2) (see Volume 2, Chapter 24), and CEF in *Chlamydomonas*. While PSII sets the redox
406 poise of the chain, only PSI participates to the CEF. Because the formation of State 2 increases

PSI antenna size, it expectedly increases PSI turnover and CEF if the later is light-limited. During the formation of state 2, a fraction of the cyt b_6f complex also moves from the PSII-enriched appressed regions of the thylakoid membrane to the stroma lamellae where PSI is localized (Vallon *et al.,* 1991). Because the co-localization of PSI and cyt b_6f and the increase of PSI antenna size coincide during state transitions, the question of their relative effects on CEF was rather difficult to address.

In 2010, Iwai and collaborators isolated a PSI-LHCI-LHCII-cyt.b_6f-PGRL1-FNR supercomplex (but lacking PGR5) under state 2. At first, the "Supercomplex model proposed CEF activation upon state 1 to state 2 transition, allowing for **i)** compartmentalization between CEF and LEF limiting their competition for the mobile electron carriers (PQ, PC, Fd) (Joliot & Joliot, 2002; Alric *et al.*, 2010) and, **ii)** tight interactions between Fd-FNR, cyt b_6f and the PQ-pool favoring fast electron transfer and high CEF rates *in vivo* (Iwai *et al.*, 2010; Joliot & Joliot, 2002; Alric *et al.*, 2010). However, those experiments could not disentangle the effect of state transitions per se, and the one of the anaerobic treatment used for their induction (which also lead to a decrease of the cellular ATP and an increase of the reducing power). When a mutant specifically devoid of state transition were used as a control, the link between state transitions and increased CEF or supercomplex formation was ruled out: increase in CEF and supercomplex formation is therefore induced by redox rather than state transitions (Lucker & Kramer, 2013; Takahashi *et al.*, 2013). Accordingly, whether it involves supercomplexes or not, electron fluxes partitioning between the LEF and the PGR5/PGRL1 CEF pathway seem to obey a competition model regulated by the stromal ATP/NADPH ratio (Alric *et al.*, 2010; Johnson *et al.*, 2014). Based on the absence of the PSI antennae subunits LHCA2 and LHCA9 in supercomplex, Steinbeck and collaborators (2018) recently proposed a mechanistical model for CEF supercomplex assembly driven by the

dissociation of these LHC under anoxia. Remarkably, Δ*lhca2* knockout mutants exhibited higher CEF rate under aerobic conditions than wildtype (WT) under either aerobic or anaerobic conditions (Steinbeck *et al.*, 2018). In any case, direct electron transfer from FNR:Fd to cyt. b_6f seems possible whether cyt b_6f forms a supercomplex with PSI or not. Furthermore, rates of CEF in supercomplexes remain to be measured experimentally as *in vivo* experiments in yeast showed that bc1-CcOx supercomplex do not display a faster kinetics of cytochrome *c* mediated electron transfer between the two complexes (Trouillard *et al.*, 2011; see however Berndtsson *et al.*, 2020).

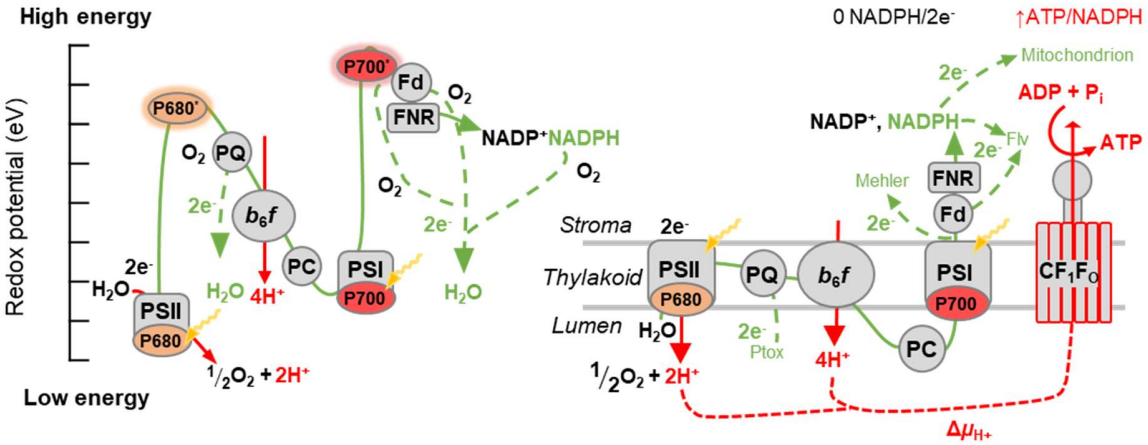
Proton pumping and regulatory role of CEF

At present we can only speculate on the path of electrons in the CEF pathway, but through the Q-cycle, it undoubtedly participates to the pumping of extra protons into the lumen. In plants, the yield of protons pumped per electron recycled around PSI is different between PGR5/PGRL1 (2 H^+/e^-) and NDH-1 (3 or 4 H^+/e^-) pathways, due to the electrogenicity of the NDH-1 complex (Strand et al 2017; Strand et al., 2019). Because NDA2 is non-electrogenic (Melo *et al.*, 2004), the NDA2 CEF pathway in *Chlamydomonas* participates to the $\Delta\mu_{H+}$ only to the same extent as the PGR5/PGRL1 pathway or the CEF based on the classical Q cycle, i.e. 2 H^+/e^-.

2.3 Water-to-water cycles

Electrons extracted from water at the PSII level can be rerouted from the LEF to various chloroplastic oxidases which utilise O_2 as electron acceptor and produce water (Curien *et al.*, 2016). This creates various AEFs linking the photo-oxidation of water into O_2 at one extremity to

449 the reduction of O_2 into water on the other, creating a water-to-water cycles (WWC) (or pseudo-
450 cyclic electron fluxes).



451 Figure 3: the Water-to-Water Cycles (WWC). A. Water-to-water cycles in the Z scheme of photosynthesis.
452 B. Representation of the different water-to-water cycles in the thylakoid membranes. Abbreviations: PSII,
453 Photosystem II; PSI, Photosystem I; b_6f, cytochrome b_6f; CF_1F_O, CF_1F_O ATPase; PC, plastocyanin; PQ,
454 Plastoquinol/Plastoquinone; Fd: ferredoxin; FNR: Ferredoxin/NADP Reductase; $\Delta\mu_{H^+}$, electrochemical
455 proton gradient.

456 **PSII-PTOX pathway**

457 By reoxidizing PQ, PTOX can divert electrons from the main transport chain. Therefore, it has
458 been suggested to have a function of safety-valve relieving excitation pressure on PSII under light
459 exposure (Cournac *et al.*, 2000; Niyogi, 2000; McDonald *et al.*, 2011). It may indeed fulfill such
460 function in organisms inhabiting harsh environments and accumulating large PTOX content
461 (Stepien & Johnson, 2009; Laureau *et al.*, 2013; Ghotbi-Ravandi *et al.*, 2019). Because electrons
462 are initially extracted from H_2O at PSII in LEF, oxidation of PQH_2 coupled to O_2 photoreduction
463 by PTOX form a WWC. However, a photoprotective role for PTOX seems contradictory with its
464 pro-oxidant activity (Heyno *et al.*, 2009; Krieger-Liszkay & Feilke, 2016) although we can not
465 discount that those ROS may serve signaling purposes (Rea *et al.*, 2018). It is also hard to reconcile

with its much slower rate compared to PSII turnover under light in *Chlamydomonas* (Houille-Vernes *et al.*, 2011) and *Arabidopsis* (Joliot & Joliot, 2002). Indeed, comparable electron transport rates measured between *Chlamydomonas* WT and *ptox2* mutants under steady illumination suggests that PTOX main function is not a photoprotective valve (Nawrocki *et al.*, 2019c). Additional observations that WT and *ptox2* mutants have similar growth rates under continuous illumination but that the mutants are severely impaired under intermittent light, prompt the authors to posit PTOX's role is to balance the PQ-pool redox state during transient light fluctuations to avoid hampering PSII driven LEF (Nawrocki *et al.*, 2019c) (see also (Houyoux *et al.*, 2011)). Slower growth of *ptox2* mutants compared to WT under intermittent light further reveals the crucial role of this pathway in the redox modulation of the PQ-pool and PSI acceptors in this condition. Heterotrophic and phototrophic metabolism intersect at the PQ-pool of which the redox state is a major electron transport regulation hub. Therefore interplay between NDA2 and PTOX influence LEF and CEF respective rates (Nawrocki *et al.*, 2019c), state transition (Houille-Vernes *et al.*, 2011) and metabolic exchanges (Wei *et al.*, 2014).

Flavodiiron proteins

Flavodiiron proteins (FLV) were first identified in cyanobacteria (Helman *et al.*, 2003) but have since been documented in all green lineages groups except angiosperms (Ilik et al, 2017). In red algae, *flv* genes have never been observed. They are also absent from most brown algae genomes (see reviews (Peltier *et al.*, 2010; Alboresi *et al.*, 2019)), even though homologs in the dinoflagellate *Symbiodinium* were reported (Roberty et al, 2014; Shimakawa *et al.*, 2021). *Chlamydomonas* synthesizes both FLVA and FLVB isoforms (homologous to FLV1 and FLV3 respectively in cyanobacteria (Peltier *et al.*, 2010)) which are believed to associate in hetero-dimer to perform a WWC by photo-reducing O_2 at PSI acceptor side (Mustila *et al.*, 2016; Chaux *et al.*,

2017). So far, only a partial structural resolution of FLV1/3 lacking the C-term domain has been revealed in *Synechocystis* (Borges *et al.*, 2019). Using *in silico* modeling, Alboresi and co-authors (2019) suggested distinct catalytic sites organisation between FLVA and FLVB (in the bryophyte *Physcomitrella patens*), enabling their action as a dimer and a possible conformational change allowing swift regulation answering to external cues like stromal redox-state. The electron donor for FLVs has been matter of debate in recent years. In *Synechocystis*, it was first proposed that FLVs may be able to receive electrons directly from NAD(P)H via a C-terminal flavin oxidoreductase-like module (Vicente *et al.*, 2002). However, FLVs were also reported to possibly interact with Fd in both *Chlamydomonas* (Peden *et al.*, 2013) and *Synechocystis* (Cassier-Chauvat & Chauvat, 2014). Most recently, evidence from comparison between re-oxidation kinetics of NADPH and of the iron sulfur cluster comprising Fd, F_X and F_AF_B (the terminal PSI acceptors) between WT and *flv1/3 Synechocystis* mutants, strongly suggested that reduced Fd-F_AF_B and not NADPH is the redox partner of FLVs (Sétif *et al.*, 2020). Interestingly, the P_{700} re-oxidation signal of *Chlamydomonas* WT, *flv* and CEF mutants (Jokel *et al.*, 2018) have similar kinetics as *Synechocystis* homologous strains (Sétif *et al.*, 2020), which possibly indicate both organisms' FLVs receive electron via reduced Fd-F_AF_B as previously proposed by Jokel and co-authors (2018).

FLVs act as a safety valve to alleviate PSI acceptor limitation and pertaining photodamage, hence accurate regulations is essential to avoid futile competition for reducing power with the CBB (Alboresi *et al.*, 2019). Interestingly, FLVs are an extremely efficient immediate electron sink upon sudden light intensity shifts (e.g. while CBB's enzymes are activating), but their activity under steady state illumination is barely detectable (Chaux *et al.*, 2017).

The Mehler reaction

512 The Mehler reaction was postulated following the observation that isolated pea thylakoids can
513 photo-reduce O_2 to peroxide (H_2O_2) (Mehler, 1951). Later seminal investigations by Asada and
514 co-workers led to the identification of $O_2^{\bullet-}$ as the initial transient product generated by charge
515 transfers from an internal PSI transporter on its acceptor side to O_2 (Asada & Kiso, 1973;
516 Takahashi & Asada, 1988). The superoxide $O_2^{\bullet-}$ anion is instantly disproportionated to H_2O_2 and
517 O_2 by the thylakoid bound superoxide dismutase and both products are released in the stroma
518 (Ogawa *et al.*, 1995). Although less toxic and a more efficient cellular messenger than $O_2^{\bullet-}$ (Rea *et*
519 *al.*, 2018), H_2O_2 remains harmful in excessive concentrations and it is scavenged in the stroma via
520 an ascorbate peroxidase using two ascorbate (AsA) molecules as electron acceptors and yielding
521 two water (thus making the Mehler reaction a WWC) and monodehydroascorbate (MDA)
522 molecules as products (Asada, 1999). Afterward, a MDA-reductase use NADPH to recycle MDA
523 back to AsA and play a role in *Chlamydomonas*' photooxidative stress tolerance (Yeh *et al.*, 2019).
524 Alternatively, MDA can spontaneously disproportionate to AsA and dehydroascorbate, which is
525 converted to a supplemental AsA via the glutathione cycle also consuming NADPH (Lin *et al.*,
526 2018).

527 The PSI pigment-protein complex encompasses two symmetrical bifurcated electron
528 chains spanning from P_{700} and converging to F_X, the phylloquinones (PhQ) A and B, located on
529 PSI heterodimer PsaA and PsaB, respectively (Volume 2, Chapter 15). Due to differences in
530 protein interactions, the electron transfer to F_X is slower from PhQ_A than from PhQ_B, with lifetimes
531 of ~300 and ~15 ns respectively, making $PhQ_A^{\bullet-}$ much more susceptible to yield electrons to
532 exogenous O_2 and generate ROS (Cherepanov *et al.*, 2017). However, a residue mutation delaying
533 even further $PhQ_A^{\bullet-}$ decay does not affect the quantum yield of PSI as electron overflow is
534 redistributed to the PhQ_B chain (Santabarbara *et al.*, 2015). Recently, Kozuleva and co-authors

(2021) proposed a novel mechanistic model for the Mehler reaction after measuring the light-dependent second-order rate of O_2 photoreduction in various *Chlamydomonas* PSI complex isolates showing mutations in different electron carriers (see other crucial works which led to the following model proposal (Kozuleva & Ivanov, 2010; Kozuleva *et al.*, 2014; Santabarbara *et al.*, 2015)). In this new model, F_AF_B participates to some extent to O_2 photoreduction but its rate saturates under moderate irradiance intensities and the major O_2 photoreduction sites is located at $PhQ_A^{\bullet-}$ (Kozuleva *et al.*, 2021). The alternative classical view is that the sole electron donor is reduced Fd and that O_2 photoreduction is favored by limited NADP+ availability (Furbank & Badger, 1983). While the Mehler reaction can partake to the $\Delta\mu_{H+}$, quantifying its contribution *in vivo* is challenging due to its transient products and interactions with other AEFs and electron flux regulation mechanisms (see conflicting views on its significance in plants (Heber, 2002; Ort & Baker, 2002)). For example, in *Chlamydomonas* exposed to high light intensity, it was, counterintuitively, reported that the Mehler action is most active when CO_2 concentration is elevated and LEF optimally functioning. When CO_2 is low, qE induction and state transition are sufficient to limit excitation pressure and H_2O_2 evolution (Roach *et al.*, 2015). Based on experimental data and the thermodynamics parameters of electron transfers within PSI redox chains, Cherepanov and co-authors (2017) developed a model predicting more that 0.3% of total electron flux could be diverted to the Mehler reaction, although it is not clear how that electron flux could be affected in the presence of FLV in *Chlamydomonas* (Chaux *et al.,* 2017). Likewise, it is likely that some, if not most, of the light-dependent O_2 uptake attributed to the Mehler reaction in previous works on *Chlamydomonas* (Peltier & Thibault, 1985, Forti & Caldiroli, 2005, Franck & Houyoux, 2008) was in fact due to the action of the then unknown FLVs (sometime called Mehler-like reaction, as FLVs do not generate H_2O_2) (see section 3.1). Simultaneous quantification

of O_2 uptake and H_2O_2 evolution in FLVs mutants will be necessary to determine substantial of an electron sink the Mehler reaction can be in *Chlamydomonas*.

Other WWCs

Other O_2 photoreduction processes can occur in the photosynthetic process, which are described in more detail in other chapters of this volume and will be only briefly described here. One of them is photorespiration (see Volume 2, Chapter 7 and 8). When O_2 rather than CO_2 enters Rubisco's catalytic site, it can trigger the oxidation of ribulose 1,5 biphosphate and generate 3-phosphoglycerate (3PGA) and 2-phophoglycolate (2PG). The subsequent suite of enzymatic reactions recycling these products (also called the C_2 cycle in reference to these two carbon intermediates) is described in Volume 2, Chapters 7 and 8. A crucial trickle-down effect of 2PG recycling to 3PGA, is that along the enzymatic reactions cycle, terminal acceptors like NADP+, NAD+, ADP and oxidized Fd are restored, fostering non-CO_2-assimilatory electron transport, which makes photorespiration a key response to harsh conditions in plants (chill, high light, CO_2 limitation) by limiting the otherwise associated overreduction of the PETC (reviewed in (Voss *et al.*, 2013)). Rubisco's oxygenase activity is unavoidable but its output tends to be limited in microalgae due to carbon concentration mechanisms (CCM) which consume ATP to gather high CO_2 density around Rubisco confined in pyrenoids (reviewed in *Chlamydomonas* in (Wang *et al.*, 2015)). The photorespiration pathway is conserved across photosynthetic phyla with two major divergences (i) reactions compartmenting in different organelles as peroxisomes and pyrenoids are not presents in all taxa, *Chlamydomonas* possesses pyrenoids but no peroxisomes and, (ii) the nature of the enzyme which oxidizes glycolate to glyoxylate. It is currently accepted that *Chlamydomonas* (and all Chlorophytes) replaced the Glycolate oxydase of plants by a glycolate dehydrogenase (GlcDH) similar to cyanobacteria (which possess both Glycolate oxydase and

581 GlcDH) (Esser *et al.*, 2014). *Chlamydomonas'* GlcDH harbors three spanning regions likely bound
582 to the mitochondrion membrane and potentially feeds electrons to the ubiquinone pool of the
583 respiratory transport chain (Beezley *et al.*, 1976; Nakamura *et al.*, 2005; Aboelmy & Peterhansel,
584 2014). Cross-talk between photorespiration and mitochondrial activity was hypothesized to
585 explain the simultaneously up-regulated transcription of their related genes under high light, which
586 could reflect enzyme synthesis for glyoxylate conversion to malate in the mitochondria (Davis *et*
587 *al.*, 2013). A study by Nakamura and co-workers (2005) strongly supported a photorespiratory
588 function to be dependent on GlcDH by characterizing a high CO_2 requiring phenotype in a
589 *Chlamydomonas* mutant with a disrupted *GlcDH* gene, very similar to typical photorespiration
590 defects in higher plants. Moreover, *GlcDH* mutants showed exacerbated glycolate excretion in the
591 medium (Nakamura *et al.*, 2005). To a lesser extent, increase in glycolate exudation was reported
592 in *Chlamydomonas* WT exposed to high light, low CO_2, in which the photorespiration pathway
593 was inhibited (Moroney *et al.*, 1986; Nakamura *et al.*, 2005) and in immobilized cells (for which
594 slowly diffusing O_2 concentration is high due to photosynthetic activity) (Garbayo *et al.*, 2005).
595 However, increased extracellular glycolate accumulation is often short-lived (Moroney *et al.*,
596 1986; Garbayo *et al.*, 2005; Günther *et al.*, 2012). This is possibly due to overcompensation by
597 enhanced CCM to limit photorespiratory carbon loss, as exemplified by steady up-regulation of
598 CCM genes compared to transient increase for photorespiration genes upon transition to low CO_2
599 concentrations (Tirumani *et al.*, 2019). In hope of developing sustain methane bioproduction from
600 glycolate, Wilhelm and co-workers (Günther *et al.*, 2012; Taubert *et al.*, 2019) demonstrated that
601 acclimating *Chlamydomonas* to low O_2/CO_2 conditions before a shift to scarce CO_2 availability
602 with addition of the CCM and GlcDH inhibitor 6-ethoxy-2-benzothiazolesulfonamide can yield
603 elevated glycolate production sustained for up to 21 d.

Another kind of WWC consists in a cooperation between photosynthesis and respiration, whereby the rerouting of the reducing power generated by the PETC towards the mitochondrial electron transfer chain eventually fuels the cytochrome oxidase (complex IV) and Alternative Oxidase. Such WWCs, described in *Chlamydomonas* and in plants (Lemaire *et al*., 1988; Krömer & Heldt, 1991) involves the malate/oxaloacetate valve as well as other metabolic shuttles, which enable the transport of reducing power from the chloroplast to the mitochondrion via the cytosol (Scheibe *et al*., 2005). Reversely, the extra ATP generated thereby in the mitochondrion can be imported back in the chloroplast through mitochondrial ATP/ADP translocators and potentially DHAP/3-PGA shuttle (Hoefnagel *et al*., 1988). The study *Chlamydomonas* mutants affected in mitochondrial respiration point to an important role of those energetic exchanges between organelles (Cardol *et al.,* 2009). Such a cooperation can restore photoautotrophic growth in a *Chlamydomonas* strain lacking the chloroplastic CF_1F_O-ATPase, by rerouting excess reducing power from the chloroplast to the mitochondrion and by importing mitochondrial ATP for CO_2 fixation (Lemaire et al., 1988).

3- An integrated and historical view of the AEFs

We now identify the importance of alternative electron transfer pathways in their common functions: poising the redox state of the electron transfer chain, playing the role of exhaust valves, or increasing the proton-motive force and ATP production. AEFs actions are integrated within a cohesive response to limit photosynthetic electron transport bottlenecks. In the last two decades, the extensive use of mutants has revealed that while interplays and redundancies are inherent to the AEFs network, it makes-up for bioenergetic resilience rather than a weakness, giving plasticity to photosynthesis to operate under changing environmental conditions and metabolic demands.

626 Some redundancy between AEFs is indeed observed, especially in the recent years between
627 CEF around PSI and WWCs mediated by FLVs. Artificial introduction of *Flv* genes in *Arabidopsis*
628 (Yamamoto *et al.*, 2016) or rice (Wada *et al.*, 2018) can partially increase growth in *pgr5* mutants.
629 In *P. patents* double mutants exhibit exacerbated defects under fluctuating light compared to single
630 *pgrl1* or *FlvA* mutants (Storti *et al.*, 2019). The redundancy of those two AEFs seems to be at place
631 in *Chlamydomonas* too, where the *pgrl1* mutants compensate deficient CEF by up-regulating FLVs
632 synthesis (Dang *et al.*, 2014). The light-dependent O_2 uptake was increased in this mutant,
633 reflecting a higher activity of FLVs together with an energetic cooperation with mitochondria
634 (Dang *et al.*, 2014). Recently, it was shown that the rate of carbon fixation in *Chlamydomonas*
635 decreases under low inorganic carbon in the double mutant of PGRL1 and FLVs compared to the
636 wild type, but not in the respective single mutants (Burlacot *et al*., 2021). This indicates that the
637 supplementary ATP demand for CO_2 concentrating mechanisms is met by the synergistic action
638 of FLVs and PGRL1-mediated CEF (Burlacot *et al*., 2021). Moreover, it is likely that accelerated
639 CEF historically reported for *Chlamydomonas* under anoxia are not only the result of a more
640 reduced photosynthetic electron chain (Alric *et al.,* 2010) but also caused by the decrease of
641 electron leaks towards FLVs (Nawrocki *et al*., 2019b).

642 This raises the question of the relative importance of the different AEFs: what are the rates of the
643 different pathways under steady-state photosynthesis? How do they change with the environmental
644 conditions (CO_2, temperature) or metabolic status of the cell? Because of this redundancy,
645 comparing mutants of the different AEFs will not be sufficient to answer such questions;
646 methodological advances in measuring and distinguishing the different electron pathways in
647 photosynthesis are needed to address these questions. During the pioneering era of photosynthesis
648 research, scientists were aiming at measuring precisely the maximum quantum yield of the

649 photosynthetic process. At that time, in order to estimate what the photochemical reactions were
650 at the molecular level, it was crucial to decide on precise values, to quantify the minimum number
651 of photons required for the production of an O_2 molecule (or the assimilation of a CO_2 molecule).
652 If we take the Emerson and Lewis value of 0.09 for the quantum yield (Emerson & Lewis, 1943),
653 it means that ~11 photons are needed (quantum requirement) to produce one molecule of O_2 or to
654 fix one molecule of CO_2. We now know that 8 electrons transferred linearly through PSI and PSII
655 are required for one O_2, that the quantum yield of PSI is close to 1 and that of PSII is about 0.8, so
656 there would remain about 1-2 reducing equivalents rerouted to "alternative pathways". Now that
657 we know the molecular players of photosynthesis, and that we have mutants of AEFs, it might be
658 worth revisiting these studies; the maximum quantum yield of photosynthesis is expected to
659 depend on the relative participation of the alternative pathways compared to the linear electron
660 transfer, and on the energy-requirement of the CO_2-concentrating mechanism.

661 Another fundamental question relates to the kinetics of activation/deactivation of the different
662 AEFs in transitory conditions. While both the PGR5/PGRL1 and FLVs help limit PSI
663 photodamage during growth under rapidly fluctuating lights, FLVs have been shown to be
664 particularly crucial because of their more rapid induction compared to CEF (Jokel et al. 2018).
665 Comparison between *pgr5*, *pgrl1* and *Flv Chlamydomonas* mutants showed that FLVs are essential
666 for survival under fluctuating light while *pgr5*/*pgrl1* growth was only slowed-down (Jokel *et al.*,
667 2018). It was therefore posited that FLVs act as a frontline defense in the first few seconds
668 following light shifts which also promote rapid ΔpH and NPQ development (Chaux *et al.*, 2017),
669 while CEF takes longer to reach sustained high functioning rates under excess light (Jokel *et al.*,
670 2018; Storti *et al.*, 2019). The best example of the sequential coordination of the different AEFs is
671 probably the dark-to-light transition in anaerobic *Chlamydomonas* cells, which has been

672 extensively studied for biohydrogen production. In these conditions, the initial photosynthetic
673 activity is entirely associated to an electron transfer from water oxidation at the PSII level toward
674 the hydrogenase, downhill of PSI, and is fully arrested in hydrogenase mutants (Godaux et al,
675 2015; Burlacot et al, 2021). This electron flow from PSII to the hydrogenase does not last more
676 than one or two minutes in the wild type and is then replaced by other AEFs that play the role of
677 relay between hydrogenase activity and CO_2 fixation. Interestingly, Godaux and collaborators,
678 who measured PSI and PSII activity at the onset of light but did not consider WWCs, concluded
679 that CEF was the relay (Godaux et al, 2015). Later, Burlacot and collaborators performed a similar
680 experiment including FLV mutants and light-induced O_2 uptake measurements (but no CEF
681 measurements) and concluded that FLVs were the major relay (Burlacot et al, 2019). Concomitant
682 measurements of CEF, H_2 production, CO_2 fixation and FLV mediated O_2 uptake at the dark-to-
683 light transitions would be useful to reconcile those reports, which however suggest that, again, a
684 synergic action of CEF and FLVs allow the transition from hydrogen production to carbon fixation.

685 How does *Chlamydomonas* relate to the great diversity of organisms performing oxygenic
686 photosynthesis, in terms of alternative photosynthetic electron routes? It is difficult to judge in
687 which species or for which kind of stress condition a particular pathway plays a more important
688 role: why should a regulatory pathway, identified as important for drought resistance in plants, be
689 conserved in oceanic algae that do not experience drought stress? Perhaps here we should
690 recognize the "chance" in the scientific approach: some AEFs have been identified and extensively
691 studied in a specific organism for practical or historical reasons. For example, the genes of the
692 chloroplast NADPH:dehydrogenase complex involved in chlororespiration were identified in
693 tobacco by homology with mitochondrial complex I (Matsubayashi *et al.,* 1987) after the whole
694 chloroplast genome sequencing in tobacco (Shinozaki *et al.,* 1986), not because of the study of

chlororespiration in tobacco. Similarly, CEF around PSI was not detected as a proton-motive force dependent on PSI excitation, but as an ATP production in spinach thylakoid membranes (broken chloroplasts) reconstituted with Fd (Tagawa *et al.,* 1963). In algae like *Chlamydomonas* (or *Chlorella*), the detection of AEFs involving O_2 as a terminal electron acceptor instead of CO_2 was favored because (i) from an experimental point of view, while it is difficult to assess the flow along a cyclic pathway where the reaction intermediates do not change concentration over time, it is relatively easy to measure the accumulation of an end-product of photosynthesis, like the net O_2 evolution induced by a continuous illumination, (ii) they are aquatic species and the detection of dissolved O_2 is easy and inexpensive, and (iii) there is no, or little photorespiration (high-CO_2 conditions or active CCM). *Chlamydomonas* is adapted to the measurements of dissolved O_2, an accurate and sensitive technique using a polarographic method (Clark electrode) independent of the mass transfer of O_2 between the liquid and the gas phase. These O_2 exchange measurements are done more cheaply and more easily in a liquid suspension of unicellular algae than on plant leaves where the gas flow through a measuring chamber must be carefully controlled, and the detection of CO_2 is often monitored using an infrared gas analyzer. In addition to the observation of the red drop (Emerson & Lewis, 1943) and enhancement effect (Brody & Emerson, 1959), which suggested the existence of two different photochemical centers, other observations, often based on the transient gush and gulps of O_2, showed the existence of pools of carriers in the electron transport chain (Joliot 1965). O_2 exchange was not only used to detect O_2 evolution but was also a pathfinder for O_2 photoreduction reactions. Perhaps the most illustrative example of the sensitivity of oxygen exchange measurements in liquid suspensions was the observation of the "Kok effect" in the green alga *Chlorella*, a break in the slope of light-response curve of the net O_2 exchange near the light compensation point (Kok 1949), suggesting an inhibition of respiration

under weak illumination, a first insight into the interaction between photosynthesis and respiration. In O_2 exchange measurements, membrane inlet mass spectrometry (Hoch *et al.,* 1963) allowed for significant progress in identifying alternative pathways using isotopic discrimination of different O_2 species. The PSII-dependent oxygen evolution from water (E_O) is monitored at a mass-to-charge ratio (m/e = 32, unlabeled $^{16}O_2$) while the oxygen uptake rate (U_O), corresponding to mitochondrial respiration, photorespiration, chlororespiration, Mehler reaction, are measured at m/e = 36 from labeled $^{18}O_2$ added to the sample before the measurement. These experiments, more specific to respiration, confirmed the light-induced changes in O_2 exchange observed earlier (Kok, 1949). In the case of *Scenedesmus*, O_2 photoreduction can compete with CO_2 reduction to the point it replaces it (Radmer & Kok, 1976), a similar phenomenon was also later observed in *Chlamydomonas* and hypothesized to contribute to the energy requirement of the CO_2-concentrating mechanism (Sültemeyer *et al.,* 1993). We now know that such large O_2 photoreduction is dependent on FLVs proteins (Chaux *et al.,* 2017) and is mostly restricted to terrestrial plants (gymnosperms), green algae (Chlorophyceae and Prasinophyceae), mosses and cyanobacteria (Peltier *et al.,* 2010). Chlororespiration was also evidenced very early on using non-labelled oxygen (Diner & Mauzerall, 1973), and later confirmed using membrane inlet mass spectrometry (Peltier *et al.,* 1987, Vermeglio *et al.,* 1990, Cournac *et al.,* 2000).

Because of this part of "chance" in the studies of AEFs in different model species, a comparative approach may be appropriate to understand the physiological role of alternative pathways in the photosynthetic diversity. It also suggests that comparisons between models or species should not be restricted to concepts and experimental results, but also take into account the materials and methods used in the work.

Table 1: Molecular actors of the Alternative Electron Flows. Gene name, CreNumber, protein name and plastid localisation, mutants name, known abiotic triggers for up-regulation of gene transcription in *Chlamydomonas* and important literature concerning the corresponding protein. One asterisks beside literature reference indicate the first observation of the pathway, two asterisks indicate the identification of the protein involved in the pathway and indicate ground-breaking results in *Chlamydomonas*. CEF PSI; cyclic electron transfer around photosystem I: WWC; water-water-cycle: HL; high light. The Mehler reaction, malate shuttle and photorespiration are excluded from WWC as they rely on a large suite of enzymes interacting with complete cell metabolism.

	Gene	CreNumber	Protein	Protein localization	Mutants	Transcription up-regulation	Literature
CEF PSI	*pgr5*	Cre05.g242400	PGR5	Stroma	*Crpgr5*, *hpm91*	-Fe	1*, 2**, 3, 4
	pgrl1	Cre07.g340200	PGRL1	Thylakoid	*pgrl1*	-Fe	1*, 5**,6, 7
	nda2	Cre19.g750547	NDA2	Thylakoid	Nda2-RNAi	-N, -S	8*, 9**,10
WWC	*flvA*	Cre12.g531900	FLVA	Stroma	*flvB*	-CO_2, -S, HL	4, 11*,12**, 13
	flvB	Cre16.g691800	FLVB	Stroma	*flvB*	-CO_2, -S, HL	4, 11*,12**, 13
	PTOX1	Cre07.g350750	PTOX1	Thylakoid	*ptox1i*		14*,15**,16
	PTOX2	Cre03.g172500	PTOX2	Thylakoid	*ptox2, ptox2i*	-N	14*,15**,16,17

Literature: 1: Arnon et al., 1954, J. Am. Chem. Soc.; 2: Munekage et al., 2002, Cell; 3: Johnson et al., 2014, Plant Physiol.; Am. Soc. Plant. Biol. 4: Jokel et al., 2018, Plant J.; 5: Dal'Corso et al., 2008, Cell; 6 : Petroustos et al., 2009 J. Biol. Chem.; 7 : Nawrocki et al., 2019b, BBA-Bioenergetics; 8 : Ogawa et al., 1991 Proc. Natl. Acad. Sci.; 9: Jans et al., 2009 Proc. Natl. Acad. Sci.; 10: Desplats et al., 2009 J. Biol. Chem.; 11: Radmer and Kok 1976 Plant Physiol.; 12: Gerotto et al., 2016 Proc. Natl. Acad. Sci.; 13: Chaux et al., 2017 Plant Physiol.; 14: Bennoun 1982 Proc. Natl. Acad. Sci.; 15: Wu et al., 1999 Plant Cell; 16: Houilles-Vernes et al., 2011 Proc. Natl. Acad. Sci.; 17: Nawrocki et al., 2019 Plant Physiol.

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