

Flavodiiron-mediated O2 photoreduction links H2 production with CO2 fixation during the anaerobic induction of photosynthesis

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3	* Corresponding author: Gilles Peltier, CEA Cadarache, 13108 Saint-Paul-lez-Durance, France
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6	Flavodiiron-mediated O ₂ photoreduction links H ₂ production with CO ₂ fixation during the
7	anaerobic induction of photosynthesis
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10 11	Adrien Burlacot ¹ , Anne Sawyer ^{2, €} , Stéphan Cuiné ¹ , Pascaline Auroy-Tarrago ¹ , Stéphanie Blangy ¹ , Thomas Happe ² , and Gilles Peltier ¹ *
12	
13	¹ Laboratoire de bioénergétique et de biotechnologie des microalgues, Institute of Biosciences and
14 15	Biotechnologies of Aix Marseille (BIAM), UMR7265, CEA, CNRS, Aix Marseille Univ, CEA Cadarache, F-13108 Saint-Paul-lez-Durance, France
16 17	² AG Photobiotechnologie, Lehrstuhl für Biochemie der Pflanzen, Fakultät für Biologie und Biotechnologie, Ruhr-Universität Bochum, Universitätsstraße 150, 44801 Bochum, Germany
18	
19	One-sentence summary: Flavodiiron proteins recycle large amounts of O ₂ during the anaerobic
20 21	induction of photosynthesis and act as a relay of hydrogenases in priming CO ₂ fixation in <i>Chlamydomonas reinhardtii</i> .
22	
23	Author contributions: A.B. and G.P. conceived the original research plans. A.B., A.S., S.C., P.A-T.,
24	and S.B. performed the experiments. A.B., A.S., T.H., and G.P. analyzed the data and wrote the
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28 29	$^{\epsilon}$ present addresses: School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia 4072, Brisbane, Australia

30 Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia

31 4072, Brisbane, Australia

32

33 Corresponding and senior author email: gilles.peltier@cea.fr

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35 Abstract (250)

36 Some microalgae, such as Chlamydomonas reinhardtii, harbor a highly flexible photosynthetic 37 apparatus capable of using different electron acceptors, including carbon dioxide (CO₂), protons, or 38 oxygen (O₂), allowing survival in diverse habitats. During anaerobic induction of photosynthesis, 39 molecular O₂ is produced at photosystem II, while at the photosystem I acceptor side, the reduction of 40 protons into hydrogen (H₂) by the plastidial [FeFe]-hydrogenases primes CO₂ fixation. Although the 41 interaction between H₂ production and CO₂ fixation has been extensively studied, their interplay with 42 O₂ produced by photosynthesis has not been considered. By simultaneously measuring gas exchange 43 and chlorophyll fluorescence, we identified an O₂ photoreduction mechanism that functions during 44 anaerobic dark to light transitions, and demonstrate that flavodiiron proteins (Flvs) are the major 45 players involved in light-dependent O₂ uptake. We further show that Flv-mediated O₂ uptake is critical 46 for the rapid induction of CO_2 fixation, but is not involved in the creation of the micro-oxic niches 47 previously proposed to protect the [FeFe]-hydrogenase from O₂. By studying a mutant lacking both 48 hydrogenases (HYDA1 and HYDA2) and both Flvs (FLVA and FLVB), we show that the induction of 49 photosynthesis is strongly delayed in the absence of both sets of proteins. Based on these data, we 50 propose that Flys are involved in an important intracellular O_2 recycling process, which acts as a relay 51 between H₂ production and CO₂ fixation.

53 INTRODUCTION

54 Chlamydomonas reinhardtii is a unicellular green alga capable of an extraordinary metabolic 55 versatility depending on the environmental conditions it encounters. As a photosynthetic organism, it 56 can grow photoautotrophically, using sunlight as an energy source, by fixing atmospheric carbon 57 dioxide (CO_2) and producing oxygen (O_2) from the photolysis of water. In the presence of organic 58 substrates such as acetate, C. reinhardtii may grow either heterotrophically or mixotrophically. Like 59 many soil microorganisms subjected to frequent shifts in O2 availability, C. reinhardtii is a facultative 60 anaerobe, able to rely either on aerobic respiration or anaerobic fermentation to oxidize reduced 61 equivalents produced by glycolysis (Catalanotti et al., 2013). During anaerobic fermentation, C. 62 *reinhardtii* cells express a set of enzymes involved in a complex network of fermentative pathways, 63 resulting in the production of acetate, formate, ethanol and hydrogen (H_2) as major end products 64 (Gfeller and Gibbs, 1984; Mus et al., 2007). Hydrogen can be produced in the dark by fermentation 65 via the pyruvate:formate lyase pathway (Noth et al., 2013), but also in the light due to the coupling of 66 photosynthesis and two plastidial [FeFe]-hydrogenases, HYDA1 and HYDA2, which use reduced 67 ferredoxin as an electron donor for the reduction of protons (Florin et al., 2001). During H_2 68 photoproduction, photolysis of water at photosystem II (PSII) supplies electrons to the photosynthetic 69 electron transport chain, resulting in the reduction of ferredoxin and production of H₂ (Hemschemeier 70 et al., 2008). This process, often referred to as the direct biophotolysis pathway, is usually transient 71 and limited by the ability of O_2 -consuming processes, such as mitochondrial respiration, to maintain 72 an anaerobic environment in the vicinity of the O_2 -sensitive [FeFe]-hydrogenases (Melis et al., 2000; 73 Hemschemeier et al., 2008; Burlacot and Peltier, 2018).

74 When dark-adapted anaerobic cultures of C. reinhardtii are exposed to sunlight, their 75 metabolism changes dramatically. During the first minutes of illumination, a transient burst of H_2 occurs (Cournac et al., 2002), then the O2 produced by photosynthesis initiates a switch from 76 77 fermentative metabolism to oxidative decarboxylation and oxidative phosphorylation by respiration 78 (Happe et al., 2002), and triggers an irreversible inhibition of the [FeFe]-hydrogenases (Erbes et al., 79 1979; Stripp et al., 2009). Kessler observed that algal species devoid of hydrogenases experience a delay in the induction of photosynthesis and proposed that H₂ photoproduction would enable algae to 80 81 oxidize photosynthetic electron acceptors and thereby activate the photosynthetic chain after anaerobic 82 incubation (Kessler, 1973). However, the selective advantage conferred by the presence of O₂-83 sensitive [FeFe]-hydrogenases in a photosynthetic organism producing O₂ remains to be demonstrated 84 (Ghysels et al., 2013).

B5 During photosynthesis, O_2 can act as an electron acceptor, either through a non-enzymatic B6 process (called the Mehler reaction) or through an enzymatic process involving flavodiiron proteins B7 (Flvs) (Peltier et al., 2010). In cyanobacteria, Flvs have been shown to catalyze the reduction of O_2 B8 into water using NADPH as an electron donor (Helman et al., 2003) and to play a critical role during B9 growth under fluctuating light regimes (Allahverdiyeva et al., 2013). In *C. reinhardtii*, Flvs have been

- 90 demonstrated to catalyze a massive and transient O₂ reduction during the induction of photosynthesis
- 91 (Chaux et al., 2017). Despite their efficiency in reducing O₂ during a light transient (Chaux et al.,
 92 2017), Flvs do not compete with CO₂ fixation (Chaux et al., 2017; Wada et al., 2017). It was proposed
- that Flvs are mainly functioning where the chloroplast stroma reaches a high reducing state (Chaux et
 al., 2017), conditions which are obtained when illuminating dark anaerobic-adapted cells (Alric,
 2010).
- In the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120, Flv3B was shown to protect the O₂-sensitive nitrogenase (Gallon, 1992) from O₂ attack (Ermakova et al., 2014). Recently, based on the continuous production of H₂ in air-grown *C. reinhardtii*, (Liran et al., 2016) it was concluded that micro-oxic niches allow the [FeFe]-hydrogenases to function in the presence of O₂ and it was proposed that Flvs may be involved in this process. However, O₂-consuming processes have been considered as minor electron sinks under H₂ production conditions (Godaux et al., 2015; Milrad et al., 2018) and, the existence of a possible O₂ reducing process remains to be demonstrated.
- 103 In this context, we questioned the role of Flv-mediated O_2 photoreduction during the anaerobic 104 induction of photosynthesis and its possible involvement in the formation of micro-oxic niches. By 105 simultaneously measuring chlorophyll fluorescence and oxygen exchange, we identified an O_2 106 reduction mechanism during a light transient in anaerobically adapted C. reinhardtii cells and further 107 demonstrate the involvement of Flvs in this process. We show that Flv-mediated O₂ reduction is 108 critical for a fast induction of CO₂ fixation, but does not protect the [FeFe]-hydrogenase from O₂ 109 inhibition by creating micro-oxic niches. We propose that Flvs act as a relay of hydrogenases to 110 promote the rapid induction of photosynthesis when O2 starts to become available as an electron 111 acceptor and the [FeFe]-hydrogenases are inhibited by O₂.
- 112

113 **RESULTS**

114 Combined gas exchanges and chlorophyll fluorescence measurements as a tool to study the photosynthetic activity of anaerobically-adapted C. reinhardtii cells. During the induction of 115 116 photosynthesis in anaerobically-acclimated cells, O2 is produced by PSII while H2 is produced by photosystem I (PSI). The presence of O2 inside the chloroplast may lead to O2 photoreduction at the 117 118 PSI acceptor side, thus resulting in O_2 recycling. In order to explore the interplay between H_2 and O_2 119 exchange during the anaerobic induction of photosynthesis, we designed a method to detect the 120 existence of such O₂ recycling in the light. Oxygen uptake in the light has been measured in the past 121 using [¹⁸O]-labeled O₂ and a membrane inlet mass spectrometer (MIMS) (Hoch and Kok, 1963), but 122 this technique cannot be used under anaerobic conditions. However, O_2 uptake rates can be inferred by 123 subtracting the net O₂ evolution from the gross O₂ produced by PSII, the latter determined from 124 chlorophyll fluorescence measurements (Kitajima and Butler, 1975). By performing simultaneous 125 measurements of chlorophyll fluorescence and gas exchange, using a pulse amplitude modulated

(PAM) fluorimeter and a MIMS with $[^{18}O]$ -labeled O_2 , respectively (Cournac et al., 2002) 126 127 (Supplemental Fig. S1A, B), we first established the relationship under aerobic conditions between the 128 chlorophyll fluorescence parameter $\Delta F/F_{M}$ ' (Supplemental Fig. S2) and the quantum yield of O₂ 129 evolution by PSII. Since our experimental set-up was not designed to measure light absorption in a 130 cell suspension, experiments were performed at similar chlorophyll concentrations, assuming a 131 constant light absorption during the time-course of the experiment. As reported previously in leaves of 132 land plants (Genty et al., 1989), a linear relationship was observed under a wide range of light 133 intensities (Fig. 1A). For the highest quantum yields (measured at low light intensities), a non-linearity 134 was observed which was unaffected by the chlorophyll concentration of the sample (Supplemental 135 Fig. S3), indicating that it likely did not result from a light heterogeneity in the cuvette. Further 136 experiments aiming at determining O₂ production by PSII from chlorophyll fluorescence 137 measurements were performed at light intensities corresponding to the linear range of the relationship 138 (Supplemental Fig. S4).

139 The PAM-MIMS set-up was then used to determine H₂ and O₂ exchange rates during a dark to 140 light transient in anaerobically-adapted C. reinhardtii cells (Fig. 1B). As previously reported (Cournac 141 et al., 2002), net H_2 and O_2 production rates rapidly increased upon illumination, with maximal values 142 being observed after about 1 min of illumination (Fig. 1B). Gross O₂ evolution rates were determined 143 from chlorophyll fluorescence measurements with the assumption that linearity in the relationship 144 established under aerobic conditions (Fig. 1A) was valid under anaerobic conditions. However, PSII 145 absorbance may vary when switching from anaerobic to aerobic conditions due to the movement of 146 light harvesting complex proteins (LHCs) between PSI and PSII, a phenomenon associated with 147 changes in the maximal fluorescence level F_M' (Wollman and Delepelaire, 1984). We were therefore 148 careful to restrict the use of the linear relationship established in Fig. 1A to the first 3 min of 149 illumination, conditions in which no major change in F_M' was observed (Supplemental Fig. S5). From the difference between the gross O_2 evolution rate (determined from chlorophyll fluorescence 150 151 measurements) and net O₂ evolution (measured by MIMS), we inferred the existence of an O₂ uptake 152 process, starting after about 50 s of illumination and reaching a maximal value after 2 min of 153 illumination, where it was estimated to be about 40% of the gross O_2 production rate (Fig. 1B and C). 154 It is noteworthy that O₂ uptake roughly started when the maximum H₂ and net O₂ production rates 155 were reached (Fig. 1B). We conclude from this experiment that O₂ recycling occurs in C. reinhardtii 156 cells upon illumination of anaerobically adapted cells and is concomitant with dramatic changes in the 157 use of electrons at the PSI acceptor side.

158

Role of Flvs in O₂ recycling. Flvs have been recently identified in *C. reinhardtii* as the main drivers of O_2 photoreduction during the induction of photosynthesis under aerobic conditions (Chaux et al., 2017). To test the possible involvement of Flvs during a light induction in anaerobically adapted cells, we performed similar experiments as in Fig. 1B in two independent *C. reinhardtii flvB* mutants

- 163 lacking both FlvB and FlvA under aerobic and anaerobic conditions (Chaux et al., 2017) 164 (Supplemental Fig. S6A). While O₂ uptake started in the wild type (WT) after about 50 s of 165 illumination and reached a maximal value after 2 min, both *flvB-208* and *flvB-308* mutants showed a 166 strongly reduced O₂ uptake during the first 2 min of illumination (Fig. 2A and B). After 3 min of 167 illumination, the O₂ uptake rate in the *flvB* mutants was much lower than in the WT (Fig. 2 B), such a 168 late increase likely reflecting a growing contribution of other O₂ uptake processes such as 169 mitochondrial respiration. We conclude from this experiment that Flvs are the major actors involved in 170 the O₂ recycling occurring upon illumination of anaerobically acclimated cells.
- 171

172 Hydrogen photoproduction is enhanced in *flvB* mutants. Hydrogen photoproduction can be limited 173 in vivo by different factors, including: i. the O₂-sensitivity of the [FeFe]-hydrogenases (Erbes et al., 174 1979), O₂ being produced in the light by PSII, and/or, *ii*. competition for electrons at the acceptor side 175 of PSI (Godaux et al., 2015). Flvs could be involved in both aspects, either by competing for electrons 176 on the PSI acceptor side, or by protecting the [FeFe]-hydrogenases from O₂ attack. Indeed, the 177 existence of intracellular micro-oxic niches protecting the [FeFe]-hydrogenases from O2 has been 178 proposed (Liran et al., 2016), and Flvs have been suggested to be involved (Liran et al., 2016; 179 Burlacot and Peltier, 2018). Hydrogen production rates of WT and *flvB* mutant strains were thus 180 compared during a dark to light transient. Enhanced H₂ photoproduction was observed in flvB mutants 181 as compared to the WT between 1 and 3 min of illumination (Fig. 3A and B), and this effect was not 182 due to a difference in initial hydrogenase activities (Supplemental Fig. S7A). The O_2 concentration in 183 the cell suspension was slightly higher in the flvB mutants compared to the WT during the first 184 minutes of the light transient (Supplemental Fig. S8). However, no difference in hydrogenase 185 inhibition was observed after 2 min of illumination in a *flvB* mutant as compared to the WT 186 (Supplemental Fig. S7B). We therefore conclude from this experiment that Flv-mediated O_2 uptake 187 competes for electrons with the [FeFe]-hydrogenases. A possible role of Flvs in the protection of the 188 [FeFe]-hydrogenases from O₂ attack could not be evidenced.

¹⁹⁰ Flv-mediated O₂ uptake primes CO₂ fixation during the transition from anaerobiosis to 191 aerobiosis. Hydrogen photoproduction has been reported to prime net photosynthesis and to allow the 192 fast induction of photosynthesis under anaerobic conditions (Ghysels et al., 2013). On the other hand, 193 Flv-mediated O₂ uptake precedes but does not influence CO₂ fixation during the induction of aerobic 194 photosynthesis (Chaux et al., 2017). To determine the extent to which Fly-mediated O_2 reduction 195 participates in the anaerobic induction of photosynthesis, we measured O_2 and CO_2 exchange in *flvB* 196 mutants. While no significant difference in net O_2 evolution and net CO_2 fixation were measured after 197 the first 3 min of illumination, a 30% decrease in both parameters was observed in both flvB mutants 198 compared to the WT (Fig. 4A and B; Supplemental Fig. S9). Remarkably, this decrease in net 199 photosynthesis was observed after H_2 production stopped, which occurred after 3-4 min of

200 illumination (Fig. 3A). We conclude from this experiment that O_2 uptake by Flvs allows a fast 201 induction of photosynthesis in anaerobically acclimated cells and primes CO_2 fixation when H_2 202 production has ceased.

203

204 Fly-mediated O₂ uptake primes CO₂ fixation even in the absence of the hydrogenase. Hydrogen 205 photoproduction has been considered as the main process enabling algae to start photosynthesis 206 following anaerobic incubation (Kessler, 1973). However, hydrogenases need at least 10 min of 207 anaerobiosis to become activated (Cournac et al., 2002) and only a restricted group of microalgae 208 possess a functional hydrogenase (Burlacot and Peltier, 2018). Given that most unicellular green algae 209 harbor Flvs (Supplemental Fig. S10), the involvement of Flvs in the start of photosynthesis after 210 anaerobic incubation in the absence of an active hydrogenase was questioned. A triple mutant 211 (hydA1/A2 flvB) lacking both hydrogenases and both Flvs was obtained by crossing two existing 212 mutants: hydA1/A2 (Meuser et al., 2012) and flvb (Chaux et al., 2017); three independent triple mutant 213 progenies (hydA1/A2 flvB 1, 2 and 3) were isolated (Supplemental Fig. S6) and further analyzed. As 214 previously shown (Ghysels et al., 2013), the lack of both hydrogenases had a dramatic effect on the 215 induction of photosynthesis upon illumination of anaerobically-acclimated cells (Fig. 5A). While 216 photosynthesis started after a 3 min illumination in the hydA1/A2 mutant, no change in the PSII yield 217 was detected in triple hydA1/A2 flvB mutants during the first 10 min of illumination (Fig. 5A). CO₂ 218 fixation was more severely affected in the triple mutants as compared to the hydA1/A2 mutant (Fig. 219 5B). We conclude from this experiment that O_2 uptake by Flvs allows a faster induction of 220 photosynthesis after anaerobic incubation even in the absence of H₂ production.

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222

223 **DISCUSSION**

224 By performing simultaneous gas exchange and chlorophyll fluorescence measurements, we show here 225 that O₂ uptake occurs at the same time as H₂ is produced upon illumination of anaerobic C. reinhardtii 226 cells. The study of flvB mutants allowed us to show the involvement of Flvs in this O₂ uptake process. 227 While different processes including H_2 production and oxidation (Gaffron and Rubin, 1942), CO_2 228 fixation, and regulatory mechanisms such as state transitions (Ghysels et al., 2013) or cyclic electron 229 flow (Godaux et al., 2015) have been shown to impact H_2 photoproduction, little attention has been 230 paid to the possible occurrence of O_2 photoreduction, this mechanism being considered as a minor 231 pathway for electrons in these conditions (Finazzi et al., 1999; Godaux et al., 2015). Here, we have 232 shown that Fly-mediated photoreduction can drive up to 40% of the total linear electron flow during 233 the induction of photosynthesis in anaerobic cells (Fig. 1B), supplying experimental evidence for the 234 existence of an important intra-cellular O₂ recycling mechanism during H₂ production. 235

- 236 Fly-mediated O₂ photoreduction primes CO₂ fixation during the transition from anaerobiosis to 237 aerobiosis. Optimal photosynthetic CO₂ fixation requires both ATP and NADPH and a fine tuning of 238 the NADPH/ATP ratio (Kramer and Evans, 2011). By reducing O₂ at the acceptor side of PSI, Flvs 239 divert electrons towards O_2 , thus limiting the NADPH supply, and therefore participate in the 240 establishment of a proton motive force which is used to produce ATP. Until now, negative effects 241 observed under fluctuating light regimes in *flv* mutants were considered to result from photoinhibition 242 (Gerotto et al., 2016; Chaux et al., 2017; Shimakawa et al., 2017), suggesting that the Flvs play a role 243 in protecting photosystems from long-term over-reduction that could lead to photoinhibition. 244 However, the loss of Flvs in algae or the expression in angiosperms of Flvs (which do not naturally 245 contain Flvs) had no effect on net photosynthesis during a dark to light transient (Chaux et al., 2017; 246 Wada et al., 2017; Yamamoto et al., 2016). This apparent discrepancy may be due to the fact that the 247 metabolic buffering capacity of cells can compensate for the loss of Flvs during steady state conditions 248 by maintaining a favorable NADHP/ATP ratio for CO₂ fixation, but that repeated light transients 249 induce a progressive imbalance in this ratio, thus resulting in PSI photoinhibition (Chaux et al., 2017). 250 Similar conditions of NADPH/ATP imbalance occur under anaerobic conditions (Clowez et al., 2015), 251 resulting in a delay in the induction of photosynthesis when Flvs are absent (Fig. 4). An alternative 252 explanation of the delayed induction in the flv mutants is that over-reduction of PSI acceptors could 253 have resulted in the production of reactive oxygen species (ROS), leading to PSI and/or PSII 254 photoinhibition. However, no difference in PSI and PSII activities was observed between WT and flv255 mutants (Supplemental Fig. S10), showing that if occurring, the production of ROS had no impact on 256 photosystem activity at this time scale. We conclude that rather than protecting photosystems from 257 photoinhibition, Flvs are involved in the priming of photosynthesis likely by rebalancing the 258 NADPH/ATP ratio. By finely tuning the NADPH/ATP ratio, Flvs protect photosystems from 259 photoinhibition in oxic conditions (Chaux et al., 2017), and enable a proper induction of CO₂ fixation 260 in anaerobiosis. This function is particularly important when the buffering capacity of cells is 261 exceeded, conditions which occur after several light transients under aerobiosis, or after a single 262 transient under anaerobiosis.
- 263

Fly-mediated O₂ reduction is a relay between H₂ production and CO₂ fixation. During the switch 264 265 from anaerobiosis to aerobiosis, H_2 photoproduction is a transient process allowing the reoxidation of 266 PSI acceptors and a fast induction of photosynthesis (Ghysels et al., 2013). Differences in the CO₂ 267 fixation rate between a flvB mutant and the WT appeared after H₂ production ceased (Supplemental 268 Fig. S9B). H₂ photoproduction therefore compensates the loss of Flvs until inhibition of the [FeFe]-269 hydrogenase by O₂. Flv-mediated O₂ reduction therefore appears to act as a relay system, supporting 270 the re-oxidation of PSI acceptors when the [FeFe]-hydrogenase is no longer functional. We have also 271 shown that Flvs act as the main drivers for the induction of photosynthesis in mutants devoid of 272 hydrogenase (Fig. 5). Interestingly, although Flvs are present in most green algae (Supplemental Fig. S11), hydrogenases are restricted to the core Chlorophytes (Meuser et al., 2011; Burlacot and Peltier, 2018). If Flvs alone may allow a transition from anaerobiosis to aerobiosis in the light in most green algae, the presence of hydrogenases may have conferred a selective advantage in specific biotopes from the core chlorophytes by allowing a faster photosynthetic induction when O_2 is not yet available.

277

278 Interplay with state transitions. During anaerobiosis, mobile light harvesting antennae are 279 phosphorylated, detach from PSII and become associated with PSI. This process, called state 280 transitions, is reversible and cells in state II (antennae associated with PSI) can return to state I 281 (antennae associated with PSII) in more oxidizing conditions. A transition to state I occurs upon 282 illumination of anaerobically adapted cells (Finazzi et al., 1999) and is of great importance for the re-283 activation of photosynthesis under these conditions (Ghysels et al., 2013). Forti and Caldiroli (Forti 284 and Caldiroli, 2005) showed that light-driven O_2 -dependent processes are involved in this process but 285 could not dissect the molecular contributors. In our study, the increase in F_M' likely due to the transition from state II to state I (Supplemental Fig. S5) occurred after H₂ production (Fig. 2A), and 286 287 when Flv-dependent O₂ uptake is maximal, highlighting the role of Flv-mediated O₂ reduction in the 288 transition to state I.

289

290 Flv-mediated O₂ reduction versus Cyclic Electron Flow (CEF). Since both Flv-mediated O₂ 291 reduction and CEF produce ATP without generating NADPH, they are expected to have similar 292 effects on photosynthesis (Shikanai and Yamamoto, 2017). Recent experiments have shown that these 293 two processes can complement each other in various organisms (Dang et al., 2014; Gerotto et al., 294 2016; Yamamoto et al., 2016; Wada et al., 2017). However, this question has not been addressed in a 295 H₂-producing context, as O₂ reduction processes have been neglected. Recently, Godaux et al., (2015) 296 observed that induction of photosynthesis in anaerobically-acclimated cells without glucose oxidase 297 was not affected by a defect in Proton Gradient Regulation Like 1 (PGRL1)-dependent CEF, which 298 indicates compensation by other mechanisms such as H₂ production or Flv-dependent O₂ reduction. 299 On the other hand, we have shown in this work that the anaerobic induction of photosynthesis is 300 impaired in *flvB* mutants. However, the maximum capacity of each alternative electron route can 301 account for up to 40% of the linear electron flow (Alric, 2014) (Fig. 1B). Our data thus suggest that 302 Flv-dependent O₂ reduction is the main alternative electron route after H₂ photoproduction upon 303 illumination in anaerobically adapted C. reinhardtii, and that in these conditions it exceeds the 304 capacity of CEF in tuning the NADPH/ATP ratio.

305

306 **Intracellular O₂ recycling.** Upon its production by PSII, O_2 can diffuse from thylakoid membranes 307 towards the extra-cellular medium. During the diffusion process, O_2 can be reduced by cellular 308 metabolic pathways, thus resulting in intra-cellular O_2 recycling. This phenomenon has been reported 309 to involve mitochondrial respiration (Lavergne, 1989), and may potentially involve other O_2 310 photoreduction pathways such as those linked to the Plastid Terminal Oxidase (PTOX) or the 311 enzymatic activities of Flvs. Because a hydrogenase deficient mutant shows PSII activity (Fig. 5A) 312 while not producing O_2 in the medium (not shown), and because this phenomenon depends on the 313 presence of Flvs (absent in a Flv and hydrogenase deficient mutant), we conclude here that Flvs take 314 part in such an intracellular O_2 recycling.

315

316 Flvs are not involved in protecting the [FeFe]-hydrogenase in micro-oxic niches. Since the report 317 that some algae can produce H_2 in the presence of atmospheric concentrations of O_2 (Liran et al., 318 2016), the possibility for intra-cellular micro-oxic niches allowing hydrogenases to be active at high 319 O₂ concentrations has been considered. However, although the molecular players involved in such 320 micro-oxic niches have not been identified so far, Flvs have been proposed as possible candidates 321 (Liran et al., 2016). We have shown here that H_2 production is not negatively impacted and is in fact 322 higher in the absence of Flvs (Fig. 2) and that the activity of the [FeFe]-hydrogenase is not affected 323 (Supplemental Fig. S5). Based on these data we conclude that at least in the experimental conditions 324 that have been tested, Flvs are not involved in the establishment of intracellular micro-oxic niches that 325 would protect the [FeFe]-hydrogenase. We instead showed that the enhancement of H_2 production in 326 *flvB* mutants likely results from an increased electron flux towards [FeFe]-hydrogenases. This directly 327 supports previous data showing that the competition for electrons at the acceptor side of PSI plays a 328 key role in H₂ photoproduction during a light transient (Godaux et al., 2015; Milrad et al., 2018). In 329 aerobic condition, Flvs have their highest activity during light transitions (Chaux et al., 2017), 330 conditions that we have explored in anaerobiosis here. However, no protective effect of the [FeFe]-331 hydrogenases could be observed under these conditions. The possibility that Flvs might protect the 332 [FeFe]-hydrogenases from O₂ in steady state low light, as proposed by (Liran et al., 2016), seems 333 rather unlikely given their reduced activity during steady state illumination (Chaux et al., 2017). 334 Further investigations will be needed to determine the possible molecular mechanisms involved in the 335 protection of [FeFe]-hydrogenases from O₂ in microalgae.

336

337 MATERIALS AND METHODS

338 Chlamydomonas strains and cultures. The C. reinhardtii WT strain CC-4533 and flvB mutants (fvlB-339 208 and flvB-308) were previously described (Chaux et al., 2017). Cells were grown 340 photoautotrophically in flasks at 25°C in HSM medium (pH 7.2) under dim light (30-40 µmol photons m⁻² s⁻¹). For some experiments (Figs. 2-4), cells were grown in photobioreactors (Dang et al., 2014; 341 Chaux et al., 2017), under a light intensity of 66 μ mol photons m⁻² s⁻¹ by bubbling air in HSM medium 342 343 (pH 6.2). C. reinhardtii double mutants devoid of both hydrogenases (hydA1/A2) and triple mutants 344 (hydA1/A2 flvB) were grown in TAP medium (Fig. 5.) Cells were cultivated under low light and 345 harvested during the exponential phase. Experiments presented throughout this manuscript were 346 performed on three independent single colony-derived lines for the WT, for each of the two strains carrying insertions in the *FLVB* gene and for each of the three *hydA1/A2 flvB* strains, thus SDs account
for standard errors of biological triplicates calculated with Prism (GraphPad Softwares, USA).

349 The mutant strain mt⁻ *flvB-308* was back-crossed twice with a 137c strain (Tolleter et al., 2011) which 350 is the parental strain of the hydA1/A2 mutant reference strain (Meuser et al., 2012). The mt⁻ flvB 351 mutant obtained was then crossed with the mt⁺ hydA1/A2 mutant. The progeny of this crossing was 352 selected based on chlorophyll fluorescence to screen for the insertion in the FLVB gene and H₂ 353 production to screen for the insertion in HYDA1 and HYDA2. We isolated three independent progenies 354 exhibiting a *flvB*-mutant-like chlorophyll fluorescence transient (Chaux et al., 2017) (Supplemental 355 Fig. S6 B) that did not produce H₂ after 1.5 h of dark anaerobic induction. Absence of the FlvB and FlvA proteins was then checked via immunodetection (Supplemental Fig. S6 B). The three triple 356 357 mutants obtained were named hydA1/A2 flvB (1, 2 and 3 respectively), CC-124 was used as a 358 reference strain for these mutants.

359 Chlorophyll fluorescence measurements. Chlorophyll fluorescence measurements were performed 360 using a pulse amplitude-modulated fluorimeter (Dual-PAM 100, Walz, Germany), the optic fiber of 361 which was coupled to a Plexiglas optic guide used as a stopper for the measuring chamber (Supplemental Figure S1). Detection pulses (10 μ mol photons m⁻² s⁻¹ blue light) were supplied at a 362 100 Hz frequency. Basal fluorescence (F_0) was measured after a 1.5h dark incubation under 363 364 anaerobiosis and for aerobic samples after 10 min of dark acclimation. Red saturating flashes (8,000 μ mol photons m⁻² s⁻¹, 600 ms) were delivered to measure F_M (in dark acclimated samples) and 365 366 then every 15 s to measure F_M' (upon actinic light exposure). PSII quantum yields were calculated as (F_M'-F_s)/F_M' according to (Genty et al., 1989). An actinic green light was chosen in order to limit light 367 368 heterogeneity in the measuring chamber, delivered by three LEDs surrounding the measuring chamber 369 and powered by a stabilized voltage generator. Anaerobic gross O₂ production was calculated 370 assuming, like in aerobic conditions, that there is a linear relationship between the quantum yield of O₂ 371 evolution and the F_V/F_M ratio (Fig. 1A). Gross O₂ evolution was then deduced linearly from the F_V/F_M 372 ratio and was minimized by the MIMS-measured net O2 evolution to overcome any initial difference 373 in PSII absorbance.

374 Membrane Inlet Mass Spectrometry (MIMS) measurements. Gas exchanges were monitored 375 inside a water-jacketed and thermoregulated (25°C) measuring chamber (modified Hansatech O₂ 376 electrode chamber) containing 1.5 mL of cell suspension. The bottom of the chamber was sealed by a 377 Teflon membrane (13 µm thickness) allowing dissolved gases to be introduced into the ion source of 378 the mass spectrometer (model Prima δB , Thermo-Fischer) through a vacuum line containing a water 379 trap cooled at -65°C. Cells were harvested from stabilized (at least 48 h) turbidostatic cultures in 380 photobioreactors or exponential growth phase cultures in flasks, then centrifuged at 450 x g for 5 min 381 and resuspended in fresh HSM medium (pH 6.2) or fresh TAP medium (pH 7.2) at a final

concentration of 20 µg chl mL⁻¹. The cell suspension was introduced into the measuring chamber 382 383 under constant stirring and the cuvette was closed with the optic guide stopper. Actinic green light was 384 supplied by three green LEDs. For anaerobic measurements, self-anaerobiosis was reached inside the measuring chamber and cells were incubated for 1.5 h or 45 min in anaerobiosis. For aerobic 385 measurements, [¹⁸O]-enriched O₂ (99% ¹⁸O₂ isotope content; Euriso-Top) was bubbled into the cell 386 suspension until approximately equal concentrations of ${}^{16}O_2$ and ${}^{18}O_2$ were reached. The mass 387 spectrometer sequentially scanned gas abundances (H₂, N₂, ¹⁶O₂, ¹⁸O₂ and CO₂) by automatically 388 adjusting the magnet current to the corresponding mass peaks (m/z = 2; 28; 32; 36; 44, respectively). 389 390 The amperometric signal collected by the spectrometer was calibrated by bubbling pure gases in a 391 cell-free solution. Gas exchange rates were determined after correction for the gas consumption by the 392 mass spectrometer assuming a first order kinetic, and normalizing H_2 , O_2 and CO_2 concentrations to 393 the N₂ concentration used as an internal reference. O₂ exchange rates were measured according to 394 (Peltier and Thibault, 1985). The apparent quantum yield of O₂ evolution was determined by dividing 395 gross O_2 evolution rates by the incident light intensity integrated over the measuring chamber.

- 396 For H/D exchange rate measurements, D_2 was bubbled into the cell suspension until saturation. Then,
- 397 the cuvette was closed and gas abundances (H₂, HD, D₂ and N₂) were recorded (m/z=2; 3; 4; 28;
- respectively). The H/D exchange rate was determined according to (Jouanneau et al., 1980).
- 399 Accession Numbers
- 400 Genes studied on this articles can be found on <u>https://phytozome.jgi.doe.gov/</u> under the loci
- 401 Cre03.g199800 (HYDA1), Cre09.g396600 (HYDA2), Cre12.g531900 (FLVA) and Cre16.g691800
- 402 (FLVB).
- 403 Sequence data from this article can be found in the GenBank data library under accession numbers
- 404 XM_001693324.1 (HYDA1), XM_001694451.1 (HYDA2), XM_001699293.1 (FLVA) and
- 405 XM_001692864.1 (FLVB).

406 Supplemental Data

- 407 Supplemental Figure S1. Experimental device used for combined measurements of gas exchange by
 408 using MIMS and chlorophyll fluorescence with a PAM.
- 409 Supplemental Figure S2. Representative chlorophyll fluorescence recordings obtained during a dark
 410 to light transient in anaerobically acclimated *C. reinhardtii* cells.
- 411 **Supplemental Figure S3.** Relationship between quantum yield of O_2 evolution and $\Delta F/F_M$ in 412 aerobiosis at a low chlorophyll concentration.
- 413 **Supplemental Figure S4.** Saturation curve of net photosynthesis with green light.
- 414 Supplemental Figure S5. Maximum fluorescence yield (F_M²) upon a shift from dark anaerobiosis to
- 415 light as a control for assessing PSII absorbance changes.
- 416 **Supplemental S6.** Characterization of the mutant *Chlamydomonas* cells.
- 417 **Supplemental Figure S7.** Hydrogenase activity assayed *in vivo* by H/D exchange.

- Supplemental Figure S8. Differences in O₂ concentration present in the MIMS chamber containing 418
- 419 flvB mutants compared to the WT during a dark to light transient in anaerobically acclimated C. <mark>reinhardtii.</mark> 420
- Supplemental Figure S9. Net O₂ and CO₂ exchange rates in the light in C. reinhardtii wild-type and 421 422 two *flvB* mutants.
- Supplemental Figure S10. PSI and PSII activities as measured by ElectroChromic Shift (ECS) upon 423 illumination of anaerobically-acclimated Chlamydomonas cells.
- 424
- Supplemental S11. Repartition of Flavodiiron proteins (Flv) and [Fe-Fe] hydrogenase on the 425 evolutionary tree of eukaryotic microalgae. 426
- 427
- 428 Supplemental Table S1. List of algae exhibiting a Flv in their genome.
- 429 430

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- 441

Figure legends 442

Figure 1. Combined measurements of gas exchange and chlorophyll fluorescence show the 443 444 existence of O₂ recycling during the first minutes of illumination of anaerobically acclimated C. *reinhardtii.* (A) Relationship between the chlorophyll fluorescence parameter $\Delta F/F_M$ and the apparent 445 446 quantum yield of gross O2 evolution established from simultaneous measurements of chlorophyll fluorescence by a PAM fluorimeter, and O_2 exchange by MIMS in the presence of [¹⁸O]-enriched O_2 . 447 448 The red line shows the linear regression for the $\Delta F/F_M$ values lower than 0.58. The chlorophyll concentration was 20 µg Chl mL⁻¹. Data were recorded after 10 minutes dark adaptation. (B) 449 Combined measurements of H₂ and O₂ exchange during a dark to light (660 μ mol photon m⁻² s⁻¹ green 450 451 light) transient in 1.5h anaerobically acclimated WT cells. Measured net O₂ and H₂ evolution rates are shown as filled circles (blue and green, respectively). Gross O2 evolution rates (black empty circles) 452 453 were determined from $\Delta F/F_M$ measurements based on the relationship established in (A), and O₂ 454 uptake rates (red empty circles) from the difference between gross and net O₂ evolution rates. Right panel: box plots show gross O₂ evolution and O₂ uptake rates determined after 3 min of illumination 455 456 (mean +/- SD, n=5 biological replicates).

457

458 Figure 2. Determination of oxygen uptake rates during the first minutes of illumination of 459 anaerobically-adapted cells. After 1.5h of dark anaerobic acclimation, algal suspensions of wild type

460 (WT, red) and two *flvB* mutants (*flvB-208* in black, and *flvB-308* in grey) were illuminated (660 µmol photons m⁻² s⁻¹ green light). Gas exchange was measured using a MIMS and PSII yields were 461 determined using a PAM. Gross oxygen evolution was determined from PSII yields. The minimal 462 463 gross oxygen uptake was calculated by subtracting the net oxygen evolution to the calculated gross 464 oxygen evolution. (A) Representative traces of minimal O₂ uptake in the WT and mutant strains. (B) 465 Mean values of minimal O₂ uptake after 1 min, 2 min and 2.5 min illumination. Shown are mean 466 values (+/-SD, n=3 for the mutants, n=6 for the WT). A star marks significant differences (Pvalue <0.05) based on ANOVA analysis (Tukey adjusted P value). 467

468

469 Figure 3. Hydrogen photoproduction in *C. reinhardtii* wild-type and two *flvB* mutants. After 1.5h 470 of dark anaerobic acclimation, wild-type (WT, red) and two flvB mutants (flvB-208 in black, and flvB-308 in grey) cell suspensions were illuminated (660 μ mol photons m⁻² s⁻¹ green light) and H₂ 471 472 production was recorded with a MIMS. (A) Representative traces of H₂ production. (B) H₂ production 473 rates during the first three minutes of illumination. Due to some variability in absolute gas exchange 474 values between biological replicates, shown data are expressed relative to the WT mean level (+/-SD, 475 n=3 for the mutants, n=6 for the WT). Stars indicate significant difference between WT and mutant 476 strains (P-value < 0.05) based on ANOVA analysis (Tukey adjusted P value).

477

478 Figure 4. Net O₂ and CO₂ exchange rates during a light transient in *C. reinhardtii* wild-type and 479 two *flvB* mutants. After 1.5h of dark anaerobic acclimation, wild-type (WT, in red) and two 480 independent *flvB* mutants (*flvB-208* in black, and *flvB-308 in grey*) cell suspensions were illuminated (660 μ mol photons m⁻² s⁻¹ green light). O₂ and CO₂ gas exchange were measured using a MIMS. (A, 481 482 B) Representative traces of net O₂ evolution and net CO₂ evolution. (C, D) Mean values of net O₂ 483 evolution (C) and net CO₂ uptake (D) rates measured at 3 min and 9 min of illumination. Due to some 484 variability in absolute gas exchange values between biological replicates, shown data are expressed 485 relative to the WT mean level (+/-SD, n=3 for the mutants, n=6 for the WT). Stars indicate significant 486 differences between the wild-type and the mutant strains (P-value < 0.05) based on ANOVA analysis 487 (Tukey adjusted P value).

488

Figure 5. Induction of photosynthesis in anaerobically adapted *C. reinhardtii* cells devoid of hydrogenases and Flvs. Chlorophyll fluorescence and gas exchange rates were recorded under illumination (660 μ mol photons m⁻² s⁻¹). (A) Operating PSII quantum yield. (B) CO₂ fixation rates measured after 9 min of illumination. Anaerobic acclimation was performed during 90 min for hydrogenase containing strains and 45 min for hydrogenase deficient strains. Shown are mean (+/-SD, n=3), a star marks a significant difference between WT and *flvB* (on the left) and triple mutants and the *hydA1/A2* mutant (on the right) based on ANOVA analysis (Tukey adjusted P value).

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