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

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7   **anaerobic induction of photosynthesis**

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19   **One-sentence summary:** Flavodiiron proteins recycle large amounts of O_2 during the anaerobic
20   induction of photosynthesis and act as a relay of hydrogenases in priming CO_2 fixation in
21   *Chlamydomonas reinhardtii*.

22

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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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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 (CO_2), protons, or

38   oxygen (O_2), allowing survival in diverse habitats. During anaerobic induction of photosynthesis,

39   molecular O_2 is produced at photosystem II, while at the photosystem I acceptor side, the reduction of

40   protons into hydrogen (H_2) by the plastidial [FeFe]-hydrogenases primes CO_2 fixation. Although the

41   interaction between H_2 production and CO_2 fixation has been extensively studied, their interplay with

42   O_2 produced by photosynthesis has not been considered. By simultaneously measuring gas exchange

43   and chlorophyll fluorescence, we identified an O_2 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 O_2 uptake. We further show that Flv-mediated O_2 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 O_2. 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 Flvs are involved in an important intracellular O_2 recycling process, which acts as a relay

51   between H_2 production and CO_2 fixation.

52

INTRODUCTION

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

When dark-adapted anaerobic cultures of *C. reinhardtii* are exposed to sunlight, their metabolism changes dramatically. During the first minutes of illumination, a transient burst of H_2 occurs (Cournac et al., 2002), then the O_2 produced by photosynthesis initiates a switch from fermentative metabolism to oxidative decarboxylation and oxidative phosphorylation by respiration (Happe et al., 2002), and triggers an irreversible inhibition of the [FeFe]-hydrogenases (Erbes et al., 1979; Stripp et al., 2009). Kessler observed that algal species devoid of hydrogenases experience a delay in the induction of photosynthesis and proposed that H_2 photoproduction would enable algae to oxidize photosynthetic electron acceptors and thereby activate the photosynthetic chain after anaerobic incubation (Kessler, 1973). However, the selective advantage conferred by the presence of O_2-sensitive [FeFe]-hydrogenases in a photosynthetic organism producing O_2 remains to be demonstrated (Ghysels et al., 2013).

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

90   demonstrated to catalyze a massive and transient O_2 reduction during the induction of photosynthesis

91   (Chaux et al., 2017). Despite their efficiency in reducing O_2 during a light transient (Chaux et al.,

92   2017), Flvs do not compete with CO_2 fixation (Chaux et al., 2017; Wada et al., 2017). It was proposed

93   that Flvs are mainly functioning where the chloroplast stroma reaches a high reducing state (Chaux et

94   al., 2017), conditions which are obtained when illuminating dark anaerobic-adapted cells (Alric,

95   2010).

96   In the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120, Flv3B was shown to protect

97   the O_2-sensitive nitrogenase (Gallon, 1992) from O_2 attack (Ermakova et al., 2014). Recently, based

98   on the continuous production of H_2 in air-grown *C. reinhardtii,* (Liran et al., 2016) it was concluded

99   that micro-oxic niches allow the [FeFe]-hydrogenases to function in the presence of O_2 and it was

100   proposed that Flvs may be involved in this process. However, O_2-consuming processes have been

101   considered as minor electron sinks under H_2 production conditions (Godaux et al., 2015; Milrad et al.,

102   2018) and, the existence of a possible O_2 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 O_2 reduction is

108   critical for a fast induction of CO_2 fixation, but does not protect the [FeFe]-hydrogenase from O_2

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 O_2 starts to become available as an electron

111   acceptor and the [FeFe]-hydrogenases are inhibited by O_2.

112

113   **RESULTS**

114   **Combined gas exchanges and chlorophyll fluorescence measurements as a tool to study the**

115   **photosynthetic activity of anaerobically-adapted *C. reinhardtii* cells.** During the induction of

116   photosynthesis in anaerobically-acclimated cells, O_2 is produced by PSII while H_2 is produced by

117   photosystem I (PSI). The presence of O_2 inside the chloroplast may lead to O_2 photoreduction at the

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 O_2 recycling in the light. Oxygen uptake in the light has been measured in the past

121   using [^{18}O]-labeled O_2 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 O_2 evolution from the gross O_2 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

126   (PAM) fluorimeter and a MIMS with [^{18}O]-labeled O_2, respectively (Cournac et al., 2002)

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 O_2

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 O_2 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 H_2 and O_2 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 O_2 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

150   the difference between the gross O_2 evolution rate (determined from chlorophyll fluorescence

151   measurements) and net O_2 evolution (measured by MIMS), we inferred the existence of an O_2 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 O_2 uptake roughly started when the maximum H_2 and net O_2 production rates

155   were reached (Fig. 1B). We conclude from this experiment that O_2 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

159   **Role of Flvs in O_2 recycling.** Flvs have been recently identified in *C. reinhardtii* as the main drivers

160   of O_2 photoreduction during the induction of photosynthesis under aerobic conditions (Chaux et al.,

161   2017). To test the possible involvement of Flvs during a light induction in anaerobically adapted cells,

162   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 O_2 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 O_2 uptake during the first 2 min of illumination (Fig. 2A and B). After 3 min of
167   illumination, the O_2 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 O_2 uptake processes such as
169   mitochondrial respiration. We conclude from this experiment that Flvs are the major actors involved in
170   the O_2 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 O_2-sensitivity of the [FeFe]-hydrogenases (Erbes et al.,
174   1979), O_2 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 O_2 attack. Indeed, the
177   existence of intracellular micro-oxic niches protecting the [FeFe]-hydrogenases from O_2 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 H_2 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 O_2 attack could not be evidenced.

189

190   **Flv-mediated O_2 uptake primes CO_2 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 O_2 uptake precedes but does not influence CO_2 fixation during the induction of aerobic
194   photosynthesis (Chaux et al., 2017). To determine the extent to which Flv-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   **Flv-mediated O_2 uptake primes CO_2 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). CO_2

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 H_2 production.

221

222

223   **DISCUSSION**

224   By performing simultaneous gas exchange and chlorophyll fluorescence measurements, we show here

225   that O_2 uptake occurs at the same time as H_2 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 O_2 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 Flv-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 O_2 recycling mechanism during H_2 production.

235

Flv-mediated O_2 photoreduction primes CO_2 fixation during the transition from anaerobiosis to aerobiosis. Optimal photosynthetic CO_2 fixation requires both ATP and NADPH and a fine tuning of the NADPH/ATP ratio (Kramer and Evans, 2011). By reducing O_2 at the acceptor side of PSI, Flvs divert electrons towards O_2, thus limiting the NADPH supply, and therefore participate in the establishment of a proton motive force which is used to produce ATP. Until now, negative effects observed under fluctuating light regimes in *flv* mutants were considered to result from photoinhibition (Gerotto et al., 2016; Chaux et al., 2017; Shimakawa et al., 2017), suggesting that the Flvs play a role in protecting photosystems from long-term over-reduction that could lead to photoinhibition. However, the loss of Flvs in algae or the expression in angiosperms of Flvs (which do not naturally contain Flvs) had no effect on net photosynthesis during a dark to light transient (Chaux et al., 2017; Wada et al., 2017; Yamamoto et al., 2016). This apparent discrepancy may be due to the fact that the metabolic buffering capacity of cells can compensate for the loss of Flvs during steady state conditions by maintaining a favorable NADHP/ATP ratio for CO_2 fixation, but that repeated light transients induce a progressive imbalance in this ratio, thus resulting in PSI photoinhibition (Chaux et al., 2017). Similar conditions of NADPH/ATP imbalance occur under anaerobic conditions (Clowez et al., 2015), resulting in a delay in the induction of photosynthesis when Flvs are absent (Fig. 4). An alternative explanation of the delayed induction in the *flv* mutants is that over-reduction of PSI acceptors could have resulted in the production of reactive oxygen species (ROS), leading to PSI and/or PSII photoinhibition. However, no difference in PSI and PSII activities was observed between WT and *flv* mutants (Supplemental Fig. S10), showing that if occurring, the production of ROS had no impact on photosystem activity at this time scale. We conclude that rather than protecting photosystems from photoinhibition, Flvs are involved in the priming of photosynthesis likely by rebalancing the NADPH/ATP ratio. By finely tuning the NADPH/ATP ratio, Flvs protect photosystems from photoinhibition in oxic conditions (Chaux et al., 2017), and enable a proper induction of CO_2 fixation in anaerobiosis. This function is particularly important when the buffering capacity of cells is exceeded, conditions which occur after several light transients under aerobiosis, or after a single transient under anaerobiosis.

Flv-mediated O_2 reduction is a relay between H_2 production and CO_2 fixation. During the switch from anaerobiosis to aerobiosis, H_2 photoproduction is a transient process allowing the reoxidation of PSI acceptors and a fast induction of photosynthesis (Ghysels et al., 2013). Differences in the CO_2 fixation rate between a *flvB* mutant and the WT appeared after H_2 production ceased (Supplemental Fig. S9B). H_2 photoproduction therefore compensates the loss of Flvs until inhibition of the [FeFe]-hydrogenase by O_2. Flv-mediated O_2 reduction therefore appears to act as a relay system, supporting the re-oxidation of PSI acceptors when the [FeFe]-hydrogenase is no longer functional. We have also shown that Flvs act as the main drivers for the induction of photosynthesis in mutants devoid of hydrogenase (Fig. 5). Interestingly, although Flvs are present in most green algae (Supplemental Fig.

273 S11), hydrogenases are restricted to the core Chlorophytes (Meuser et al., 2011; Burlacot and Peltier,

274 2018). If Flvs alone may allow a transition from anaerobiosis to aerobiosis in the light in most green

275 algae, the presence of hydrogenases may have conferred a selective advantage in specific biotopes

276 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

286 transition from state II to state I (Supplemental Fig. S5) occurred after H_2 production (Fig. 2A), and

287 when Flv-dependent O_2 uptake is maximal, highlighting the role of Flv-mediated O_2 reduction in the

288 transition to state I.

289

290 **Flv-mediated O_2 reduction versus Cyclic Electron Flow (CEF).** Since both Flv-mediated O_2

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 H_2-producing context, as O_2 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 H_2 production or Flv-dependent O_2 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 O_2 reduction is the main alternative electron route after H_2 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 O_2 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   O_2 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 H_2 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 O_2 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 O_2 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
341   m^{-2} s^{-1}). For some experiments (Figs. 2-4), cells were grown in photobioreactors (Dang et al., 2014;
342   Chaux et al., 2017), under a light intensity of 66 µmol photons m^{-2} s^{-1} by bubbling air in HSM medium
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

347 carrying insertions in the *FLVB* gene and for each of the three *hydA1/A2 flvB* strains, thus SDs account

348 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 H_2

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 H_2 after 1.5 h of dark anaerobic induction. Absence of the FlvB and

356 FlvA proteins was then checked via immunodetection (Supplemental Fig. S6 B). The three triple

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

362 (Supplemental Figure S1). Detection pulses (10 µmol photons m^{-2} s^{-1} blue light) were supplied at a

363 100 Hz frequency. Basal fluorescence (F_0) was measured after a 1.5h dark incubation under

364 anaerobiosis and for aerobic samples after 10 min of dark acclimation. Red saturating flashes

365 (8,000 µmol photons $m^{-2} s^{-1}$, 600 ms) were delivered to measure F_M (in dark acclimated samples) and

366 then every 15 s to measure F_M' (upon actinic light exposure). PSII quantum yields were calculated as

367 (F_M'-F_s)/F_M' according to (Genty et al., 1989). An actinic green light was chosen in order to limit light

368 heterogeneity in the measuring chamber, delivered by three LEDs surrounding the measuring chamber

369 and powered by a stabilized voltage generator. Anaerobic gross O_2 production was calculated

370 assuming, like in aerobic conditions, that there is a linear relationship between the quantum yield of O_2

371 evolution and the F_V/F_M ratio (Fig. 1A). Gross O_2 evolution was then deduced linearly from the F_V/F_M

372 ratio and was minimized by the MIMS-measured net O_2 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 O_2

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 under constant stirring and the cuvette was closed with the optic guide stopper. Actinic green light was 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 measurements, [^{18}O]-enriched O_2 (99% $^{18}O_2$ isotope content; Euriso-Top) was bubbled into the cell suspension until approximately equal concentrations of $^{16}O_2$ and $^{18}O_2$ were reached. The mass spectrometer sequentially scanned gas abundances (H_2, N_2, $^{16}O_2$, $^{18}O_2$ and CO_2) by automatically adjusting the magnet current to the corresponding mass peaks (m/z = 2; 28; 32; 36; 44, respectively). The amperometric signal collected by the spectrometer was calibrated by bubbling pure gases in a cell-free solution. Gas exchange rates were determined after correction for the gas consumption by the mass spectrometer assuming a first order kinetic, and normalizing H_2, O_2 and CO_2 concentrations to the N_2 concentration used as an internal reference. O_2 exchange rates were measured according to (Peltier and Thibault, 1985). The apparent quantum yield of O_2 evolution was determined by dividing gross O_2 evolution rates by the incident light intensity integrated over the measuring chamber.

For H/D exchange rate measurements, D_2 was bubbled into the cell suspension until saturation. Then, the cuvette was closed and gas abundances (H_2, HD, D_2 and N_2) were recorded (m/z=2; 3; 4; 28; respectively). The H/D exchange rate was determined according to (Jouanneau et al., 1980).

Accession Numbers

Genes studied on this articles can be found on https://phytozome.jgi.doe.gov/ under the loci Cre03.g199800 (HYDA1), Cre09.g396600 (HYDA2), Cre12.g531900 (FLVA) and Cre16.g691800 (FLVB).

Sequence data from this article can be found in the GenBank data library under accession numbers XM_001693324.1 (HYDA1), XM_001694451.1 (HYDA2), XM_001699293.1 (FLVA) and XM_001692864.1 (FLVB).

Supplemental Data

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

Acknowledgements

Figure legends

Figure 1. Combined measurements of gas exchange and chlorophyll fluorescence show the existence of O_2 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 quantum yield of gross O_2 evolution established from simultaneous measurements of chlorophyll fluorescence by a PAM fluorimeter, and O_2 exchange by MIMS in the presence of [^{18}O]-enriched O_2. 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) Combined measurements of H_2 and O_2 exchange during a dark to light (660 µmol photon m^{-2} s^{-1} green light) transient in 1.5h anaerobically acclimated WT cells. Measured net O_2 and H_2 evolution rates are shown as filled circles (blue and green, respectively). Gross O_2 evolution rates (black empty circles) were determined from $\Delta F/F_M$ measurements based on the relationship established in (A), and O_2 uptake rates (red empty circles) from the difference between gross and net O_2 evolution rates. Right panel: box plots show gross O_2 evolution and O_2 uptake rates determined after 3 min of illumination (mean +/- SD, n=5 biological replicates).

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

(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 determined using a PAM. Gross oxygen evolution was determined from PSII yields. The minimal gross oxygen uptake was calculated by subtracting the net oxygen evolution to the calculated gross oxygen evolution. (A) Representative traces of minimal O_2 uptake in the WT and mutant strains. (B) Mean values of minimal O_2 uptake after 1 min, 2 min and 2.5 min illumination. Shown are mean 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).

Figure 3. Hydrogen photoproduction in *C. reinhardtii* wild-type and two *flvB* mutants. After 1.5h 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 μmol photons m^{-2} s^{-1} green light) and H_2 production was recorded with a MIMS. (A) Representative traces of H_2 production. (B) H_2 production rates during the first three minutes of illumination. Due to some variability in absolute gas exchange values between biological replicates, shown data are expressed relative to the WT mean level (+/-SD, n=3 for the mutants, n=6 for the WT). Stars indicate significant difference between WT and mutant strains (P-value < 0.05) based on ANOVA analysis (Tukey adjusted P value).

Figure 4. Net O_2 and CO_2 exchange rates during a light transient in *C. reinhardtii* wild-type and two *flvB* mutants. After 1.5h of dark anaerobic acclimation, wild-type (WT, in red) and two independent *flvB* mutants (*flvB-208* in black, and *flvB-308 in grey*) cell suspensions were illuminated (660 μmol photons m^{-2} s^{-1} green light). O_2 and CO_2 gas exchange were measured using a MIMS. (A, B) Representative traces of net O_2 evolution and net CO_2 evolution. (C, D) Mean values of net O_2 evolution (C) and net CO_2 uptake (D) rates measured at 3 min and 9 min of illumination. Due to some variability in absolute gas exchange values between biological replicates, shown data are expressed relative to the WT mean level (+/-SD, n=3 for the mutants, n=6 for the WT). Stars indicate significant differences between the wild-type and the mutant strains (P-value < 0.05) based on ANOVA analysis (Tukey adjusted P value).

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 μmol photons m^{-2} s^{-1}). (A) Operating PSII quantum yield. (B) CO_2 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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