Flavodiiron Proteins Promote Fast and Transient O$_2$ Photoreduction in *Chlamydomonas*$_{1}^{[OPEN]}$ 

Frédéric Chaux,$^{2}$ Adrien Burlacot,$^{2}$ Malika Mekhalfi, Pascaline Auroy, Stéphanie Blangy, Pierre Richaud, and Gilles Peltier$^{3}$

CEA, CNRS, Aix-Marseille Université, Institut de Biosciences et Biotechnologies Aix-Marseille, UMR 7265, Laboratoire de Bioénergétique et Biotechnologie des Bactéries et Microalgues, CEA Cadarache, Saint-Paul-lez-Durance, F-13108 France

**ORCID IDs:** 0000-0001-7434-6416 (A.B.); 0000-0002-2226-3931 (G.P.).

During oxygenic photosynthesis, the reducing power generated by light energy conversion is mainly used to reduce carbon dioxide. In bacteria and archaea, flavodiiron (Flv) proteins catalyze O$_2$ or NO reduction, thus protecting cells against oxidative or nitrosative stress. These proteins are found in cyanobacteria, mosses, and microalgae, but have been lost in angiosperms. Here, we used chlorophyll fluorescence and oxygen exchange measurement using [18O]-labeled O$_2$ and a membrane inlet mass spectrometer to characterize *Chlamydomonas reinhardtii* *flvB* insertion mutants devoid of both FlvB and FlvA proteins. We show that Flv proteins are involved in a photo-dependent electron flow to oxygen, which drives most of the photosynthetic electron flow during the induction of photosynthesis. As a consequence, the chlorophyll fluorescence patterns are strongly affected in *flv* mutants during a light transient, showing a lower PSI operating yield and a slower nonphotochemical quenching induction. Photoautotrophic growth of *flv* mutants was indistinguishable from the wild type under constant light, but severely impaired under fluctuating light due to PSI photo damage. Remarkably, net photosynthesis of *flv* mutants was higher than in the wild type during the initial hour of a fluctuating light regime, but this advantage vanished under long-term exposure, and turned into PSI photo damage, thus explaining the marked growth retardation observed in these conditions. We conclude that the *C. reinhardtii* Flv participates in a Mehler-like reduction of O$_2$, which drives a large part of the photosynthetic electron flow during a light transient and is thus critical for growth under fluctuating light regimes.

**Oxygenic photosynthesis**, by reducing CO$_2$ into biomass and using water as an electron donor, is responsible for the major entry of carbon into ecosystems. During oxygenic photosynthesis, electrons originating from water splitting at PSII are transferred to PSI, which reduces NADP$^+$ into NADPH at its acceptor side. Electron transfer reactions generate as well a proton motive force (pmf) that drives the ATP synthesis. ATP and NADPH are then used to fuel the CO$_2$ photoreduction cycle. Although the main flow of electrons generated by oxygenic photosynthesis is used for CO$_2$ assimilation, it was early recognized that a significant part of electrons is diverted to molecular oxygen (Mehler, 1951; Radmer and Kok, 1976). Different mechanisms of light-dependent O$_2$ consumption have been described in the chloroplast, including direct O$_2$ photoreduction at the PSI acceptor side (also called Mehler reactions), the oxygenase activity of Rubisco (also called photorespiration), or the reduction of O$_2$ by the plastidial terminal oxidase PTOX (also called chlororespiration). The respective contribution of these different pathways has been a matter of debate and may considerably vary depending on the experimental conditions and according to the organisms considered (Badger et al., 2000). The physiological function of the different O$_2$ photoreduction pathways has been controversial, since they have been alternatively viewed as futile pathways resulting in a waste of energy, as protective mechanisms avoiding photooxidative damage, or as a mean to (re)-equilibrate the balance between reducing and phosphorylating powers through pseudocyclic photophosphorylations (Badger, 1985; Allen, 2003). In cyanobacteria, flavodiiron proteins catalyze NADPH-dependent reduction of O$_2$ at the PSI acceptor side (Vicente et al., 2002; Helman et al., 2003; Allahverdiyeva et al., 2013). The *Synechocystis* sp. PCC 6803 genome harbors four *Flv* genes, two of them (*Flv1* and *Flv3*) encoding proteins that form a functional heterodimer catalyzing O$_2$ photoreduction (Helman et al., 2003) and allowing growth under fluctuating light (Allahverdiyeva et al., 2011). The two others (*Flv2* and *Flv4*) code for proteins protecting PSI from over-reduction (Zhang et al., 2009, 2012) by using a...
yet-unidentified electron acceptor (Allahverdiyeva et al., 2015). Flo genes have been conserved in microalgae, mosses, and gymnosperms, but are notably absent from angiosperms genomes.

In Arabidopsis (Arabidopsis thaliana), the capacity to grow under fluctuating light depends on a strict regulation of the photosynthetic electron flow by the proton gradient, so-called photosynthetic control, which depends on the activity of cyclic electron flow (CEF) around PSI mediated by PGR5 and PGR1 (Munekage et al., 2002; DalCorso et al., 2008; Suorsa et al., 2012). In cyanobacteria, the PGR5/PGR1-dependent CEF is not functional, and it was proposed that growth under fluctuating light relies on the presence of Flv1 and Flv3 that would act as an electron sink preventing overreduction of PSI acceptors and production of reactive oxygen species (Allahverdiyeva et al., 2013). Algae and mosses harbor both Flvs and PGRL1/PGR5 CEF components (Tolleter et al., 2011; Dang et al., 2013). With the aim to better understand the function of Flvs in microalgal photosynthesis, and particularly to determine whether oxygen is the electron acceptor of Flvs in microalgal photosynthesis, and especially to understand the function of Flvs in microalgae, mosses, and gymnosperms, but are notably absent from angiosperms genomes.

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With the aim to better understand the function of Flvs in microalgal photosynthesis, and particularly to determine whether oxygen is the electron acceptor of Flv and what part of the electron flow is diverted toward oxygen photoreduction during photosynthesis, we study here C. reinhardtii flvb insertion mutants of the Chlamydomonas Library Project (CLiP) library (Li et al., 2016) devoid of both FlvB and FlvA proteins. By performing photosynthetic gas exchange measurements using [18O]-labeled O2 and a membrane inlet mass spectrometer (MIMS), we show that O2 photoreduction is strongly decreased in flvb mutants, particularly during the induction phase of photosynthesis. We conclude that FlvA and FlvB proteins are involved in a Mehler-like O2 photoreduction, which massively drives electrons to O2 during the induction phase of photosynthesis.

**RESULTS**

**Identification and Preliminary Characterization of Four Independent C. reinhardtii flvb Mutants**

To investigate the function of Flvs in the unicellular alga C. reinhardtii, we searched for insertion mutants in the Flvb (Cre16.g691800) gene region in the CLiP library (https://www.chlamylibrary.org; Li et al., 2016). We obtained four putative flvb mutants, three holding insertion of the paromomycin resistance cassette in introns and one with a possible large deletion (Fig. 1A; Supplemental Table S1). All strains grew normally on Tris-acetate-phosphate or minimal medium under constant low light (40 μmol photons m−2 s−1). Insertion was confirmed by performing PCR on genomic DNA of flvb-14, flvb-208, and flvb-308 (Supplemental Fig. S1). We did not, however, succeed in mapping the insertion in the putative flvb-21 mutant, possibly due to atypical genomic rearrangement(s) that may occur with cassette insertion (Li et al., 2016). Immunodetection was then performed on total whole-cell protein extracts by using antibodies directed against recombinant FlvB and FlvA proteins (Fig. 1, B and C). FlvB and FlvA were not detected in the three independent flvb-21, flvb-208, and flvb-308 strains, while a low level was detected in flvb-14 (hereafter called knockdown mutant). The difference in protein accumulation observed in flvb-208 and flvb-14 is surprising, since insertions are located in the same intron (Fig. 1A). This might be due to a differential effect on intron splicing depending on the location of the insertion in the intron. Similar patterns of accumulation were previously observed for the Flv1 protein in the Synechocystis flv3 mutant (Allahverdiyeva et al., 2013) and for the FlvA protein in the P. patens flvb mutant (Gerotto et al., 2016), which were interpreted by the existence of a functional heterodimer between Flv1 and Flv3 in Synechocystis and between FlvA and FlvB in P. patens.

**Chlorophyll Fluorescence Transients Are Strongly Affected under High Light in flvb Mutants**

To determine how electron transfer reactions are affected in flvb mutants, we recorded chlorophyll fluorescence transients under different light intensities. Under low actinic light (25 μmol photons m−2 s−1), no difference could be recorded between flvb mutants and the CC-4533 strain, hereafter referred to as the wild type (Fig. 2A). However, upon exposure to higher light intensities (100 or 500 μmol photons m−2 s−1), a marked difference was observed between flvb mutants and the wild type, the fluorescence signal transiently reaching a much higher value in the mutants than in the wild type (Fig. 2, B and C). The PSI operating yield measured after 20 s of actinic illumination was much lower in flvb mutants than in the wild type, but the difference partially vanished after 5 min of light exposure (Fig. 3). A smaller effect was observed in flvb-14, which accumulates low levels of FlvB and FlvA (Fig. 2B; Supplemental Fig. S2). Interestingly, relaxation of chlorophyll fluorescence to a steady-state level after a saturating flash was slower in the flvb mutants than in the wild-type strain (Supplemental Fig. S3), indicating that Flv proteins are functional during brief light transients. We conclude from these chlorophyll fluorescence measurements that Flv proteins are involved in photosynthetic electron transfer reactions, particularly
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**During the induction phase of photosynthesis under moderate or high illumination.**

Flv Proteins Interact with the Photosynthetic Electron Transport Chain at the Level of PSI Acceptors

To better characterize the site of interaction between Flv proteins and the photosynthetic electron transport chain, we first used DBMIB, a photosynthetic electron flow inhibitor acting at the Q₀ site of the cytochrome b₅f complex. Upon addition of DBMIB, the chlorophyll fluorescence level rapidly rose to the maximal fluorescence level (Fₘ) with similar light induction curves in the wild type and *flvB* mutants (Fig. 4), thus indicating that Flv proteins interact with the photosynthetic electron transport chain downstream the cytochrome b₅f. We then used oxidized methyl viologen (MV), which efficiently accepts electrons at the PSI acceptor side. The chlorophyll fluorescence induction curve of the wild type was only slightly affected by MV (Fig. 4). In contrast, the strong chlorophyll fluorescence increase observed in the *flvB* mutant was suppressed by the MV treatment, thus indicating that the site of action of Flv is located downstream the action site of MV, which is at the PSI acceptor.

*Figure 1.* Characterization of independent *C. reinhardtii* mutants carrying an insertion in the *flvB* gene. A, Four mutant strains from the CLiP (www.chlamylibrary.org) harboring putative insertions of the paromomycin resistance cassette in the *flvB* locus (Cre16.g691800) were characterized. Exons, introns, and untranslated regions are shown as red boxes, black lines, and gray boxes, respectively. Genomic sequences flanking the insertion cassette of the four putative *flvB* mutant strains obtained from the CLiP Web site were used to design primers (F1/R2 and F8/R9) to confirm the location of insertion. B and C, Immunoanalysis of FlvB and FlvA protein amounts were carried out using antibodies produced against recombinant FlvB (B) and FlvA (C) proteins, respectively. A cytochrome f antibody was used as loading control.
side level. Altogether, these observations also show that Flv proteins are active in highly reducing conditions and that its activity is decreased when the reducing pressure is low.

**Oxygen Photoreduction Is Strongly Reduced in flvB Mutants**

We then measured O₂ exchange during light transients using a MIMS and [¹⁸O]-labeled O₂ (Fig. 5). This technique allows discriminating unidirectional fluxes of O₂ in the light: i, gross O₂ evolution, which represents O₂ produced by PSII, and ii, O₂ uptake in the light, which consists in different mechanisms such as O₂ photoreduction and mitochondrial respiration. In such experiments, O₂-consuming processes take up all O₂ species present in the medium including [¹⁸O]-labeled O₂, while photosynthesis will essentially produce non-labeled O₂ from water-splitting at PSII. When light was switched on, the O₂ uptake rate strongly increased in the wild type, and then progressively declined during the light period (Fig. 5A). In the flvB-21 mutant, the O₂ uptake rate measured in the light was much lower than in the wild type and remained constant throughout the light period (Fig. 5B), thus showing that Flv proteins are involved in O₂ photoreduction. The O₂ uptake rate remained constant throughout the light period in flvB mutants and was close to the O₂ exchange rate measured in the dark, which is mainly due in *Chlamydomonas* to persistent mitochondrial respiration (Peltier and Thibault, 1985b). Therefore, the large O₂ uptake component observed in the wild type during the first minutes of illumination is mainly due to the activity of Flv proteins. Gross O₂ evolution was also reduced in the flvB-21 mutant as compared to the wild type, indicating that in the wild type Flv-mediated O₂ photoreduction promotes an extra-electron flow from PSII to O₂. Similar effects were observed in the three independent flvB mutants (flvB-21, flvB-208, and flvB-308), while an intermediary effect was observed in the flvB-14 knockdown strain (Fig. 5, C and D). Dark O₂ uptake rates measured in the different strains before and after the illumination period did not show significant differences (Supplemental Fig. S4). The net O₂ evolution rate measured after 5 min of illumination, which reflects net photosynthesis due to CO₂ reduction, was not much affected in the mutant as compared to the wild type (Fig. 5D). This shows that Flv-dependent O₂ photoreduction does not efficiently compete with CO₂ fixation during steady-state photosynthesis. Note that after 5 min of illumination, O₂

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**Figure 2.** Chlorophyll fluorescence measurements during dark to light transients in *C. reinhardtii* wild type (WT) and flvB mutant strains. Cells were grown in HSM medium under low light (40 μmol photon m⁻² s⁻¹) and harvested during exponential phase. Chlorophyll fluorescence measurements were performed using pulse amplitude modulated fluorimeter in the dark (black boxes) and under red actinic light (white boxes) of different intensities: 25 μmol photon m⁻² s⁻¹ (A), 100 μmol photon m⁻² s⁻¹ (B), and 500 μmol photon m⁻² s⁻¹ (C). Saturating flashes were supplied when indicated by red vertical lines. Data are normalized on initial Fₐ measurements and traces of mutant strains are shifted few seconds to the right for clarity.
uptake rates remained slightly higher in the wild type as compared to flvB mutant strains (Fig. 5D), indicating that the Flv-mediated electron flow to O₂ also contributes under steady-state illumination. We conclude from these experiments that O₂ photoreduction primes and replaces CO₂ fixation during the induction phase of photosynthesis in C. reinhardtii, as was previously reported in Scenedesmus obliquus and Anacystis nidulans (Radmer and Kok, 1976), and we further establish that Flv proteins are involved in this phenomenon.

Growth of flvB Mutants Is Delayed under Fluctuating Light Conditions Due to PSI Impairment

The strong effect observed in flvB mutants during the induction phase of photosynthesis and the growth delay previously reported in Δflv cyanobacterial mutants grown under fluctuating light (Allahverdiyeva et al., 2011) prompted us to analyze growth performances under repeated light changes. When C. reinhardtii cells were grown under 5 min low light (50 μmol photons m⁻² s⁻¹) and 1 min high light (500 μmol photons m⁻² s⁻¹), a strong growth retardation was observed in the three flvB mutant strains (flvB-21, flvB-208, and flvB-308) as compared to the wild type, while an intermediary effect was observed in the flvB-14 strain (Fig. 6A). This drastic effect on growth did not, however, lead to cell death, since growth recovery was observed during a subsequent growth period under continuous light (Supplemental Fig. S5). In contrast, no growth difference could be observed between mutant and wild-type strains under constant illumination, even under high light intensity (800 μmol photons m⁻² s⁻¹; Fig. 6A).

To understand the mechanism underlying growth retardation, we performed PSII and PSI activity measurements at different time points during exposure to fluctuating light conditions (Fig. 6B). Maximal PSI activity was slightly decreased (by about 20%) in the flvB mutant upon 48 h of fluctuating light exposure, but a drastic decrease of the PSI activity (about 80%) was observed in the mutant (Fig. 6B). Immunoanalysis of PSII and PSI subunits, respectively PsbA and PsaD, revealed no major change in the PsbA subunit amount, but a large decrease in PsaD in the flvB mutant upon 24 h of exposure to fluctuating light (Fig. 6C). We conclude from this experiment that repeated transitions from low to high light induce PSI photoinhibition in the absence of Flv-mediated electron flow.

Defect in Flv Has Ambivalent Effects on Photosynthesis

To better characterize the function of Flv proteins during photosynthesis, we measured photosynthetic O₂ exchange in cells during exposure to a fluctuating light regime (Fig. 7). Net photosynthesis was higher in flvB mutant strains compared to the wild type, with especially high rates observed in the flvB-21 strain (Fig. 7A). This effect was slightly more pronounced under high light exposure (Fig. 7B). As a result, the net O₂ exchange rate in the flvB-21 strain was significantly lower compared to the wild type (Fig. 7C). To further explore the effect of Flv on photosynthesis, we analyzed the efficiency of light absorption by the photoreceptors in the two strains. The conversion of absorbed light energy into electrons was significantly lower in the flvB-21 strain (Fig. 7D). Additionally, we observed that the efficiency of light absorption by the PSII antenna was significantly lower in the flvB-21 strain (Fig. 7E). These results indicate that the Flv proteins play a role in the light harvesting process and in the efficiency of light-to-electron conversion.

Figure 3. Light dependence of the PSII yield in the wild type (WT) and flvB mutant strains. Upon 10-min dark adaptation under constant air bubbling, algal suspensions of wild type and flvB mutant strains were introduced in the cuvette of a pulse amplitude-modulated fluorimeter. PSII yields were measured after 20 s (dotted lines) or 5 min (plain lines) of illumination at different light intensities. Shown are the mean values (±SD, n = 3).

Figure 4. Effect of DBMIB and MV on chlorophyll fluorescence transients measured in wild type (WT) and flvB mutant strains. Samples were placed in the dark (black box) in the presence of 1 μM DBMIB, 1 mM MV, or without addition of any chemicals. Chlorophyll fluorescence was measured during a transient dark to light (100 μmol photon m⁻² s⁻¹) transient. Saturating flashes were supplied when indicated by red vertical lines. Shown are the traces representative of n = 2 independent measurements. Fluorescence data have been normalized on F₀.
mutant cells during the first 30 min of a fluctuating light exposure as compared to the wild type (Fig. 7A), but the difference vanished after 1 h, and a negative effect was observed upon 4 h of fluctuating light exposure (Fig. 7C), in accordance with the inhibition of PSI (Fig. 6). In contrast, the O₂ uptake rate measured in the light was initially much lower in the flvB mutant than in the wild type (Figs. 5, A and B, and 7, B and D), but progressively increased in the mutant until reaching a similar level as in the wild type (Fig. 7D). This indicates that during the time-course of an exposure to a fluctuating light regime, a Flv-independent O₂ uptake process was progressively triggered in the mutant. Therefore, depending on the duration of a fluctuating light exposure, the loss of Flv proteins leads either to a positive or a negative effect on net photosynthesis, the positive effect being accompanied by a decrease in the O₂ uptake (mediated by Flv) and the negative effect being accompanied by a progressive increase in an O₂ uptake component. The induction of this Flv-independent, light-dependent O₂ uptake phenomenon likely results in ROS production, thus explaining growth retardation and PSI photoinhibition observed under long-term exposure (Fig. 6).

Nonphotochemical Quenching Induction Is Affected in flvB Mutant Strains

To better understand the impact of the Flv-mediated electron flow to O₂ on photosynthesis, we measured the nonphotochemical quenching (NPQ) induction in high light-adapted cells (Fig. 8A). During a dark to light transient, NPQ rapidly rose in the wild type to reach a steady-state level. NPQ induction was slower in the flvB mutant, but reached a similar level after 1 to 2 min of illumination (Fig. 8A). In C. reinhardtii, NPQ relies on...
two factors: accumulation of the LHCSR3 protein and lumen acidification (Peers et al., 2009; Bonente et al., 2011). Since both wild-type and flvB mutant strains accumulated similar levels of the LHCSR3 protein (Fig. 8B), the slower induction of NPQ is likely due to a slower acidification of the lumen in flvB mutants as compared to the wild type. To further investigate effects on the proton gradient, we measured electrochromic carotenoid shift (ECS). In the flvB mutant, the pmf was decreased by one third and the ΔpH component by ~50% as compared to the wild type (Fig. 8C), the ratio between ΔΨ and ΔpH components being unaffected (Supplemental Fig. S6). Moreover, time constant of ECS decay indicates that membrane conductivity to protons (gH) is not altered in the flvB mutant, whereas proton flux (nH = pmf * gH) is lower (Fig. 8D), suggesting a lower rate of ATP synthesis in the mutant during the induction of photosynthesis. We conclude from this experiment that the photosynthetic electron flow to O₂ mediated by Flv proteins participates in the wild type to the establishment of pmf that allows fast induction of the NPQ and higher ATP production during a light transient (Fig. 9A).

DISCUSSION

Flv Proteins Are Involved in O₂ Photoreduction during a Light Transient

By characterizing four independent insertion mutants of C. reinhardtii in the Flvb gene region affected in the accumulation of FlvB and FlvA proteins, we show here that Flv are involved in O₂ photoreduction at the acceptor side of PSI. Forty years ago, Radmer and Kok used [18O]-labeled O₂ and mass spectrometry to show that in Scenedesmus and Anacystis, O₂ photoreduction primes and replaces CO₂ fixation during the induction of photosynthesis (Radmer and Kok, 1976), but mechanisms of O₂ uptake in the light. Under steady-state conditions, persistent mitochondrial respiration in the light (Peltier and Thibault, 1985b) and a small contribution of photorespiration could be detected in C. reinhardtii (Peltier and Thibault, 1985a), the latter operating in microalgae at a much lower rate than in C₃ plants (Badger et al., 2000). We have shown here that Flv-mediated O₂ photoreduction drives most of the photosynthetic electron flow during the induction phase of photosynthesis in wild-type strains, when CO₂ assimilation has not started yet. Flv activity appears to be restricted to highly reducing conditions, which might result from a lower affinity to NADPH than the Calvin cycle enzymes, as recently proposed (Shikanai and Yamamoto, 2017).
Chlamydomonas FlvA/FlvB Proteins Function in a Similar Manner as Cyanobacterial Flv1/Flv3

Phylogenetic analysis of the Flv family has shown that algal and mosses FlvB proteins are homologous to cyanobacterial Flv3 and Flv4, while FlvA proteins are homologous to cyanobacterial Flv1 and Flv2 (Zhang et al., 2009; Peltier et al., 2010). In Synechocystis PCC6803, Flv1 and Flv3 have been proposed to catalyze O₂ photoreduction into water without generation of ROS, and protect PSI from inhibition under fluctuating light (Allahverdiyeva et al., 2013). Based on O₂ photoreduction measurements in Δflv1 and Δflv3 cyanobacterial mutants, it was concluded that Flv3 function as a hetero-dimer with Flv1 (Helman et al., 2003; Allahverdiyeva et al., 2011). However, a functional Flv3 homo-oligomer may also be formed, as deduced from the overproduction of Flv3 in Synechocystis (Mustila et al., 2016). On the other hand, Flv2 and Flv4 protect PSII by a different mechanism and using a yet-uncharacterized electron acceptor (Zhang et al., 2012; Bersanini et al., 2014). From our data, we conclude that C. reinhardtii FlvA and FlvB are involved in O₂ photoreduction at the PSI acceptor side and therefore likely functions in a similar manner as cyanobacterial Flv1 and Flv3. Because the presence of Flv proteins prevents PSI photodamage, it is most likely that O₂ is fully reduced into water without production of ROS, as previously proposed for cyanobacteria (Helman et al., 2003). Based on the fact that FlvA could not be detected in flvB knockout mutants (Fig. 1C), we conclude that C. reinhardtii FlvA and FlvB proteins form in vivo a functional heterodimer, as previously proposed for Flv1 and Flv3 in Synechocystis (Helman et al., 2003; Allahverdiyeva et al., 2013) and FlvA and FlvB in P. patens (Gerotto et al., 2016).

Flv Proteins Are Involved in a Pseudocyclic Electron Flow to O₂ and Participates in the Establishment of a pmf

By mediating an electron flow from water to O₂ Flv proteins are involved in a pseudocyclic electron flow
and therefore participates in the generation of a pmf (Fig. 8, C and D). In addition to its role in ATP synthesis, the pmf triggers two main mechanisms, the qE-type NPQ (LHCSR3-dependent) and the photosynthetic control, which participate in the regulation of the photosynthetic electron flow and protect PSI from acceptor-side limitations and PSI photoinhibition (Chaux et al., 2015, 2017). The CEF also participates in the establishment of a pmf, and its role in triggering both NPQ and the photosynthetic control has been established (Munekage et al., 2002; Joliot and Johnson, 2011; Tolleter et al., 2011; Suorsa et al., 2012). As shown here by the delayed induction of NPQ (Fig. 7A) measured in flvB mutants during a light transient, the Flv-mediated pseudocyclic electron flow to O2 is involved in the rapid establishment of NPQ (Fig. 8A).

In *Chlamydomonas*, Flv-Mediated Electron Flow to O2 Has Ambivalent Effects on Photosynthesis

Although no effect of Flv deficiency was observed on photosynthesis and growth under continuous illumination, ambivalent effects were observed upon exposure to fluctuating light. During a short-term exposure (<1 h) of fluctuating light, net photosynthesis was enhanced in flvB mutants as compared to the wild type. However, this advantage vanished thereafter, net photosynthesis being strongly decreased in the mutant (Fig. 7C). To understand such an ambivalent effect, we need to consider the different factors involved in the limitation of photosynthesis. Photosynthetic CO2 fixation requires both NADPH and ATP, and a fine tune in the supply of both energy sources is needed for an optimal functioning of photosynthesis (Kramer and Evans, 2011). In other words, photosynthetic CO2 fixation can be either limited by the NADPH supply or by the ATP supply. The Flv-mediated electron flow from PSII to O2 has two effects on photosynthesis, one is to divert electrons toward O2, therefore limiting the NADPH supply and the other to generate pmf, which may either be used to produce ATP and down-regulate the photosynthetic electron flow. Lack of Flv would therefore favor NADPH supply by two means: by avoiding the loss of electrons toward O2 and by lowering the down-regulation of photosynthetic electron flow. A positive effect of a Flv defect on photosynthesis would therefore indicate that photosynthesis is limited by the supply of reducing power (high ATP/NADPH ratio), when cells switch from a continuous to a fluctuating light regime. However, in conditions where ATP is lacking (low ATP/NADPH ratio), the defect in Flv would enhance the disequilibrium by lowering ATP and increasing NADPH.

The ambivalent effect of Flv may therefore reveal the existence of different limitation regimes of photosynthesis (NADPH-limited versus ATP-limited) under fluctuating light conditions, a progressive switch from a NADPH-limited regime toward an ATP-limited regime occurring upon long-term exposure to fluctuating light.

**Figure 8.** NPQ induction and proton motive force (pmf) measurements in the wild type (WT) and flvB-21 mutant. A, For NPQ measurements, cells were exposed to 200 μmol photon m⁻² s⁻¹ for 4 h to induce accumulation of the LHCSR3 prior to chlorophyll fluorescence measurements. The wild type and flvB-21 mutant were then exposed to 500 μmol photon m⁻² s⁻¹ in the PAM cuvette (white box) and NPQ determined from chlorophyll fluorescence measurements. NPQ values are the mean (±SD, n = 3) in the wild type (red symbols) and flvB-21 mutant (black symbols). B, Immunodetection of LHCSR3 protein in experimental conditions as described in (A) in wild type (red) and different flvB mutants. C, Different components of the pmf (∆ψ and ∆pH) were determined from ECS measurements in wild type (red) and flvB-21 mutant (gray) from similar experiments as described in (Supplemental Fig. SSA). D, Membrane proton conductivity (gH+ and proton flow (νH+) were determined from ECS measurements. Numbers above bars (C and D) represent uncorrected P values as determined by ANOVA using Fischer’s L...
In the absence of Flv, cells would produce more NADPH than strictly needed to make photosynthesis working at its optimal regime and would progressively accumulate an excess of reducing power. The 1-h retardation in the appearance of the negative effect would be due to the buffering capacity of intracellular metabolic pools and electron sinks. When the excess of NADPH produced exceeds the buffering capacity, overaccumulation of reducing power would result in the production of ROS and in turn to PSI photoinhibition (Fig. 6, B and C). A similar scenario based on a progressive disequilibrium between NADPH and ATP production was recently proposed to explain the delay between PSI photoinhibition and CO2 when a double C. reinhardtii mutant deficient in CEF and qE was exposed to a sudden light increase (Chaux et al., 2017).

Compensation between Flvs-Mediated O2 Photoreduction and PGR1L/PGR5-Dependent CEF

In the absence of CEF, Arabidopsis pgr5 and pgr1 mutants are prone to PSI photoinhibition and severe growth retardation under high light (DalCorso et al., 2008; Munekage et al., 2008). The wild-type phenotype was restored in the Arabidopsis pgr5 mutant by the expression of P. patens FlvA and FlvB proteins, thus showing that artificially rewiring the photosynthetic electron flow to O2 through Flvs can compensate deficiency in CEF (Yamamoto et al., 2016). In Synechocystis, which lacks PGR5-PGRL1 CEF, deletion of Flv1 and Flv3 results in a severe decrease (about 60%) in net photosynthesis under constant high light (Allahverdiyeva et al., 2013). This strongly contrasts with C. reinhardtii, where no decrease in net photosynthesis was observed under constant light in flvB mutants (Fig. 5D). This difference may result from a compensation by the PGR1L/PGR5-dependent CEF, which like pseudocyclic electron flow generates extra-PMF and extra-ATP during photosynthesis. Indeed, deficiency in CEF in C. reinhardtii was partially compensated by an increase in Flv-dependent O2 photoreduction (Dang et al., 2014). Therefore, Flv-dependent O2 photoreduction and CEF appear as partially redundant mechanisms in microalgae, both able to supply extra-ATP during steady-state photosynthesis.

Under fluctuating light conditions, growth is severely impaired in C. reinhardtii flvB mutants (Fig. 6), as previously observed in Synechocystis Δflv1 and Δflv3 mutants (Allahverdiyeva et al., 2013). This shows that in C. reinhardtii the PGR5-PGRL1 CEF is not able to compensate for the loss of Flvs under fluctuating light. In contrast, Arabidopsis growth under fluctuating light relies on the PGR1L/PGR5-mediated CEF, which protects PSI from photoinhibition in the absence of Flvs (Suorsa et al., 2012), indicating that the overall process of CEF may be more efficient in angiosperms than in algae.

CONCLUSION

While the role of Flv in driving O2 photoreduction in a photosynthetic organism has been first demonstrated in cyanobacteria (Helman et al., 2003), recent studies performed in the moss P. patens (Gerotto et al., 2016) and liverwort (Shimakawa et al., 2017), and mainly based on chlorophyll fluorescence measurements, concluded that Flv drive alternative electron flow and protect PSI particularly under fluctuating light. However, there was no experimental evidence until now of an Flv-catalyzed O2 photoreduction in microalgae (Curien et al., 2016). Here, by using 18O-labeled O2 and MIMS, we clearly establish that Flv are functional in microalgae and involved in O2 photoreduction. Moreover, quantification of O2 photoreduction rates showed that Flv massively drives electrons toward O2 during the induction of photosynthesis. Positive or negative effect of Flv observed on net photosynthesis depending on the fluctuating light conditions, may supply an experimental basis to understand why Flvs were conserved in some species like gymnosperms and algae and discarded in angiosperms. Finally, the loss of Flv in angiosperms could be related to the existence of a more efficient CEF.

MATERIALS AND METHODS

Chlamydomonas Cultures

The Chlamydomonas reinhardtii wild-type strain CC-4533 and flvB mutants were obtained from the CLiP (Li et al., 2016). Upon reception, strains were plated on Tris-acetate-phosphate medium and streaked until exhaustion. After a 1-week growth in the dark, three single-clone derived colonies were randomly chosen for conservation and subsequent characterization for each strain. For further liquid culture experiment, cells were grown in flasks at 25°C in HSM medium under dim light (30–40 μmol photon m−2 s−1). Unless otherwise stated, experiments presented throughout this manuscript were performed on three single colony-derived lines for the wild type and for each of the four strains carrying insertions in the flvB gene; thus, SDs account for sfs of biological triplicates calculated with Prism (GraphPad Software).

Spot Tests

Cells were harvested during exponential phase and resuspended in fresh HSM to 2, 10, or 20 μg chlorophyll mL−1. Ten-microliter drops were spotted on plates and exposed to different light regimes. Homogeneous light was supplied by a panel of 400 cool-white LEDs (1 cm distant of each other) and placed 33 cm above plates for growth tests. Temperature was maintained at 25°C at the level of plates by means of fans. The LED panel was powered via a capacitor voltage transformer giving a direct current with a variable voltage between 0 and 54 V. Voltage was monitored via an Arduino UNO microcontroller board to obtain the appropriate light fluctuation regimes.

PCR Procedures

Total DNA was extracted using Chelex kit (Sigma-Aldrich) as described (Dang et al., 2014). Putative insertions were confirmed in three of the four strains by PCR using Takara LA Taq DNA polymerase with GC buffer (Clontech). The following set of primers was designed according to Cre16.g691800 gene sequence (Phytozome v5.5; www.phytozome.jgi.doe.gov): F1: 5′-GAGGCCATGCGACCTAGCC for FLVA and R2: 5′-GCACGGCACCATCTCCGACCTAGCC for FLVB first intron region, F8: 5′-GAGGCATGCGACCTAGCC for FLVA and R9: 5′-CACCCTCGG-AGTAGGTGACCCAGTGGTCG for FLVB seventeenth intron region. Cycles were as follows: 2 min at 94°C / 35 cycles: 20 s at 94°C, 20 s at 64°C, 2 min at 72°C / 1 min at 72°C. PCR products were separated on 1% (w/v) agarose gels.

Production of FlvB and FlvA Antibodies

Synthetic FLVA and FLVB genes were cloned respectively into the pLIC7 and the Champion pET151 Directional TOPO (Invitrogen) expression vectors,
allowing the production of a recombinant FLVA fused to TEV-cleavable His-tagged Escherichia coli thioredoxin and the recombinant FLVB protein fused to a N-ter (Hisbol10). Production was performed in the E. coli BL21 Star (DE3) strain grown at 37°C in TB medium. Induction was initiated at an OD600 = 0.6 by adding 0.5 μl isopropyl β-D-thiogalactoside (Sigma-Aldrich). Following overnight incubation at 25°C, cells were centrifuged and pellets resuspended in a lysis buffer containing an antiprotease inhibitor cocktail (SigmaFast tablet), 0.25 mg mL−1 lysozyme, and 10 μg mL−1 DNase. Following incubation (30 min at 4°C), cells were sonicated and centrifuged (12,000g for 30 min at 4°C). Crude protein extracts were loaded on a His-Trap HP column (GE Healthcare) and eluted with 250 mM imidazole. The thioredoxin fused to FLVA was cleaved off with overnight incubation with TEV protease. FLVA and FLVB fractions were loaded onto a HiPrep 26/60 Sephadex 5-200 HR size-exclusion column (GE Healthcare) and recovered with 10 mM Tris, pH 8.0, buffer containing 300 mM NaCl. The protein peaks containing recombinant FLVA in one case and the (Hisbol−FlvB in the other case were controlled for purity on SDS-PAGE and concentrated using a Amicon-Ultra device (Millipore). Polyclonal antibodies against FlvA and FlvB were raised in rabbits (ProteoGenix).

Immunodetection

Cells (10–15 mL) were harvested from liquid cultures and centrifuged at 3,000g for 2 min. Pellets were then frozen in liquid nitrogen and stored at −20°C until use. Pellets were resuspended in 400 μL 1% SDS and then 1.6 mL acetonitrile (−20°C) was added. After overnight incubation at −20°C, samples were centrifuged (14,000 rpm, 10 min). Supernatant was removed and used for chlorophyll quantification using SAFAS UVmc spectrophotometer (SAFAS). Pellets were resuspended to 1 μg chlorophyll mL−1 in LDS in the presence of NuPAGE reducing agent (ThermoFischer) and loaded on 10% PAGE Bis-Tris gel. A secondary antibody was added for at least 1 h before detection with a Fuji imaging system (Millipore). Polyclonal antibodies against FlvA and FlvB were raised in rabbits (ProteoGenix).

Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence measurements shown in Figures 2, 4, 6, and 7 were performed using a pulse amplitude-modulated fluorimeter (Dual-PAM 100) upon 5 min dark-adaptation under continuous stirring. Detection pulses (10 μmol photon m−2 s−1 blue light) were supplied at a 100-Hz frequency. Basal fluorescence (F0) was measured in the dark prior to the first saturating flash. Red saturating flashes (6,000 μmol photon m−2 s−1, 600 ms) were delivered to measure Fm (in the dark) and Fm′ (in the light). PSII maximum yields were calculated as (Fm′−F0)/Fm′. In the experiment shown in Figure 4, DBMIB and MV (Sigma-Aldrich) were added 1 min prior to measurements at final concentrations of 1 μmol and 1 μmol, respectively. For NPQ measurements (Fig. 7), cells were grown in high light (200 μmol photon m−2 s−1) for 24 h to induce LHCSR3 accumulation. Measurements were then performed under 500 μmol photon m−2 s−1 for 10 min and NPQ calculated as (Fm′−F0)/Fm′. Chlorophyll fluorescence measurements shown in Figure 3 were performed using a Joliot-type spectrofluorimeter (JTS 10; BioLogic). Cells were harvested from liquid cultures, centrifuged (3,000 rpm, 2 min), and resuspended in a 3-mL glass cuvette in a buffer containing 20 μl Ficoll buffered with 20 mM HEPES (pH 7.2). Upon 10 min in the dark, fluorescence was obtained after nonactinic detection pulses of blue light given in the dark (F1 parameter), during green light saturating flashes (4,000 μmol photon m−2 s−1, 250 ms; F1 and F1′ parameters), and during green light illumination at 40, 190, 370, 640, 1,220, and 1,800 μmol photon m−2 s−1 (F parameter). Maximal PSII yield was calculated from (Fm′−F0)/Fm′ before illumination, and PSII yield for each light intensity was calculated from (Fm′−F0)/Fm′.

Absorption Change Measurements

P700 absorption and carotenoid ECS were measured at 705 and 520 nm, respectively, using a JTS-10 (BioLogic). Cells were harvested from flask cultures, centrifuged at 3,000 rpm for 2 min, and resuspended in a 3-mL glass cuvette in a buffer containing 20% Ficoll buffered with 20 μl HEPES (pH 7.2). For P700 absorbance change measurements, PSII was inhibited by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (10 μM final concentration), and PSII acceptor side limitations were prevented by addition of oxidized MV, 10 μM final concentration). Measurements were carried out in the dark to reach a baseline level and in response to saturating red light (1,200 μmol photons m−2 s−1). P700w was accumulated within 1 to 2 s. Concentration of oxidizable P700w was calculated from steady-state (−5 s) relative absorbance levels and molar extinction coefficient (A580nm−A640nm)/−502.3 (Hiyama and Ki, 1972; Joliot and Joliot, 2005). P700 absorption changes measured in the absence of MV showed similar results (not shown). For ECS measurements, low light-grown cells were dark-adapted for 5 min in an open cuvette manually bubbled with a syringe (aerobic conditions) and rapidly introduced in the JTS sample holder (BioLogic). ECS was measured during 5 s under orange light (1,170 μmol photons m−2 s−1) and normalized to the ECS value measured in response to 6 s single-turnover flash. Total pmf values (ECS) were calculated from the difference between the ECS value after 5 s in light and the minimum value obtained 300 to 500 ms after light was switched off (Supplemental Fig. S6A). ΔV and ΔpH components of the pmf were calculated according to the difference between ECS at maximal ECS value 3 s to 5 s after the light was switched off. Using Prism (GraphPad Software), data points of the last seconds of illumination and 0 to 500 ms of dark relaxation were fitted to the “Plateau followed by one-phase decay” function, from which time constants were extracted. Membrane proton conductivity gSt was calculated as the inverse of the time constant and proton flow νt (as the product of pmf and gSt) (Cruz et al., 2005).

MIMS Measurements

O2 exchanges were monitored using a water-jacketed, thermoregulated (25°C) reaction vessel coupled to a mass spectrometer (model Prima DB; Thermo Electron) through a membrane inlet system (Tollette et al., 2011). The cell suspension (1.5 mL) was placed in the reaction vessel, and bicarbonate (4 mM final concentration) was added to reach CO2 saturation. [18O]enriched O2 (99% 18O isotope content; Euriso-Top) was bubbled at the top of the suspension until reaching approximately equal concentrations of 16O2 and 18O2. Upon closure of the reaction vessel, O2 exchanges were measured during a 2-min dark period, then light was switched on (800 μmol photons m−2 s−1). Isotopic O2 species (16O2/18O2) (m/z = 36) and [16O3/18O4] (m/z = 32) were monitored, and O2 exchange rates were determined as described previously (Radmer and Kok, 1976).

Statistical Analysis

Statistical significance was assessed by ANOVA using GraphPad Prism (GraphPad Software). P values were computed by multiple comparison tests using Fischer’s LSD test (uncorrected P values) or using Tukey corrections for large datasets (adjusted P values).Shown are the P values or the grouping of strains into statistical families according to P values. We defined the statistical significance cutoff as 0.05 (5%).

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Wild-type and mutant strains from the CLiP used in this study.

Supplemental Figure S1. PCR characterization of the insertion region in the three flvB mutants flvB-14, flvB-208, and flvB-308.

Supplemental Figure S2. Light dependence of PSI yield in wild type and flvB mutant strains.

Supplemental Figure S3. Chlorophyll fluorescence kinetics in response to a saturating flash in the wild type and flvB mutant strains.

Supplemental Figure S4. Measurements of dark- and light-induced oxygen uptake rates in the wild type and flvB mutant strains.

Supplemental Figure S5. Growth recovery of flvB mutants after long exposure to fluctuating light.

Supplemental Figure S6. ECS measurements in the wild-type and flvB-21 mutant strains.