

# Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light

Yagut Allahverdiyeva<sup>a</sup>, Henna Mustila<sup>a</sup>, Maria Ermakova<sup>a</sup>, Luca Bersanini<sup>a</sup>, Pierre Richaud<sup>b,c,d</sup>, Ghada Ajlani<sup>e</sup>, Natalia Battchikova<sup>a</sup>, Laurent Cournac<sup>b,c,d,f</sup>, and Eva-Mari Aro<sup>a,1</sup>

<sup>a</sup>Laboratory of Molecular Plant Biology, Department of Biochemistry and Food Chemistry, University of Turku, FI-20014, Turku, Finland; <sup>b</sup>Commissariat à l'Énergie Atomique, Direction des Sciences du Vivant, Institut de Biologie Environnementale et de Biotechnologie, Laboratoire de Bioénergétique et Biotechnologie des Bactéries et Microalgues, Commissariat à l'Énergie Atomique Cadarache, F-13108 Saint-Paul-lez-Durance, France; <sup>c</sup>Centre National de la Recherche Scientifique and <sup>d</sup>Aix Marseille Université, Unité Mixte de Recherche 7265 Biologie Végétale et Microbiologie Environnementales, F-13108 Saint-Paul-lez-Durance, France; <sup>e</sup>Institute of Biology and Technology Saclay, Centre National de la Recherche Scientifique, Commissariat à l'Énergie Atomique Saclay, F-91191 Gif-sur-Yvette, France; and <sup>f</sup>Institut de Recherche pour le Développement, Unité Mixte de Recherche Ecologie Fonctionnelle et Biogéochimie des Sols and Agro-Écosystèmes, F-34060 Montpellier, France

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Cyanobacterial flavodiiron proteins (FDPs; A-type flavoprotein, Flv) comprise, besides the  $\beta$ -lactamase-like and flavodoxin domains typical for all FDPs, an extra NAD(P)H:flavin oxidoreductase module and thus differ from FDPs in other Bacteria and Archaea. *Synechocystis* sp. PCC 6803 has four genes encoding the FDPs. Flv1 and Flv3 function as an NAD(P)H:oxygen oxidoreductase, donating electrons directly to O<sub>2</sub> without production of reactive oxygen species. Here we show that the Flv1 and Flv3 proteins are crucial for cyanobacteria under fluctuating light, a typical light condition in aquatic environments. Under constant-light conditions, regardless of light intensity, the Flv1 and Flv3 proteins are dispensable. In contrast, under fluctuating light conditions, the growth and photosynthesis of the  $\Delta flv1(A)$  and/or  $\Delta flv3(A)$  mutants of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 become arrested, resulting in cell death in the most severe cases. This reaction is mainly caused by malfunction of photosystem I and oxidative damage induced by reactive oxygen species generated during abrupt short-term increases in light intensity. Unlike higher plants that lack the FDPs and use the Proton Gradient Regulation 5 to safeguard photosystem I, the cyanobacterial homolog of Proton Gradient Regulation 5 is shown not to be crucial for growth under fluctuating light. Instead, the unique Flv1/Flv3 heterodimer maintains the redox balance of the electron transfer chain in cyanobacteria and provides protection for photosystem I under fluctuating growth light. Evolution of unique cyanobacterial FDPs is discussed as a prerequisite for the development of oxygenic photosynthesis.

Mehler reaction | membrane inlet mass spectrometry | photorespiration | terminal oxidases

Flavodiiron proteins [FDPs; also called A-type flavoproteins (Flv)] comprise a family of proteins widespread among strict or facultative anaerobic Bacteria and Archaea but are also present in some anaerobic pathogenic protozoa, green algae, mosses, and lycophytes (1, 2). FDPs have been suggested to function as homodimers or homotetramers on detoxification of NO and/or O<sub>2</sub> during nitrosative and/or oxidative stress. Some of the FDPs show high affinity toward NO and others to O<sub>2</sub>, whereas some enzymes catalyze both reactions but with different efficiency. FDPs share a common core comprised of two redox centers: the N-terminal  $\beta$ -lactamase-like domain with nonheme catalytic diiron center and the C-terminal flavodoxin-like domain with a flavin mononucleotide cofactor. Cyanobacterial FDPs have acquired an extra C-terminal flavin-reductase domain, thereby enabling the oxidation of NAD(P)H and reduction of a substrate within the same protein.

Cyanobacterial FDPs can be grouped into two clusters and seem to function as heterodimers with one monomer from each cluster (2). Some  $\alpha$ -cyanobacteria and ancient eukaryotic species possess only two putative FDPs, whereas N<sub>2</sub>-fixing heterocystous

cyanobacteria possess six FDPs. Unicellular non-N<sub>2</sub>-fixing cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) has four genes encoding FDPs, Flv1–Flv4. Flv1 and Flv3 types of FDPs are present in all cyanobacteria, but the Flv2 and Flv4 FDPs are present only in  $\beta$ -cyanobacteria. We have recently shown that the Flv2/Flv4 heterodimer functions in photoprotection of photosystem (PS) II (2, 3). The Flv1 and Flv3 proteins have been shown to function in the light-induced O<sub>2</sub> uptake in Mehler-like reaction that does not produce reactive oxygen species (ROS) (4, 5). It was shown that ~20–30% of electrons released from water splitting by PSII are directed to O<sub>2</sub> via Flv1 and Flv3 proteins upon high-light conditions. Under Ci-deprivation conditions the Flv1/Flv3 proteins, in cooperation with the photorespiratory pathway, might flux up to 60% of electrons to O<sub>2</sub>, functioning as a powerful electron sink (5). Elimination of the Flv1 and Flv3 proteins seems to have no detrimental effect on the growth and wellbeing of the *Synechocystis* cells under standard growth conditions (2, 4, 5). Impaired acclimation ability of the  $\Delta flv3$  mutant has been reported only upon a shift of cells from slow growth mode (low light, air-level CO<sub>2</sub>), to rapid growth mode (standard growth light, 5% CO<sub>2</sub>) and also in combination with the  $\Delta gcvT$  mutation that renders cells deficient in the photorespiratory 2-phosphoglycolate metabolism (6). Unlike *Synechocystis*, filamentous, N<sub>2</sub>-fixing, heterocystous cyanobacterium *Anabaena* sp. PCC 7120 (hereafter, *Anabaena*) has six genes encoding FDPs. Whereas *flv1A*, *flv3A*, *flv2*, and *flv4* in *Anabaena* are highly homologous with genes from *Synechocystis*, the *flv1B* and *flv3B* genes encoding heterocyst-specific Flv1B and Flv3B proteins are likely to be involved in N<sub>2</sub> metabolism (7).

Here we addressed the impact of FDPs upon growth of *Synechocystis* and *Anabaena* under fluctuating light (FL) conditions. We have recently identified in higher plants two proteins, the Proton Gradient Regulation 5 (PGR5) and the STN7 kinase of the light-harvesting complex II proteins, as absolute requirements for successful growth of plants under FL (8, 9). Because of light harvesting systems different from those in plant chloroplasts, cyanobacteria lack the gene coding for the STN7 kinase, and their *pgr5*-like gene is only a distant homolog of the plant *pgr5* gene. As natural light conditions experienced by photosynthetic microorganisms in aquatic environments might be extremely variable in the light intensity, it is conceivable that

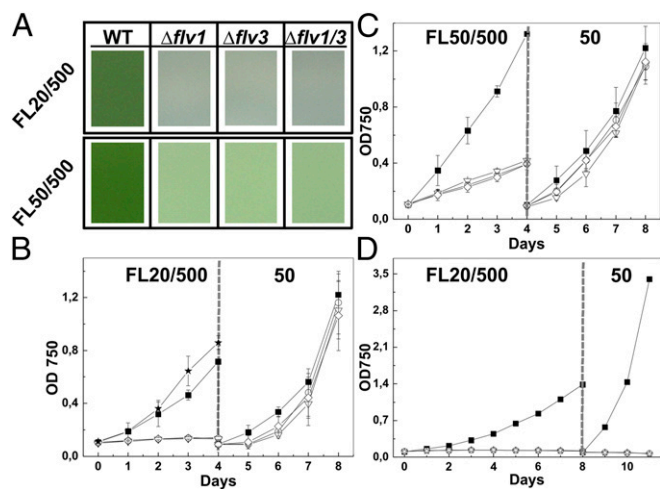
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<sup>1</sup>To whom correspondence should be addressed. E-mail: evaaro@utu.fi.

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**Fig. 1.** Growth phenotype of *Synechocystis* WT and mutant cells. (A) Cells were grown under FL 20/500 or 50/500 regime for 4 d. (B) Cells were grown under FL 20/500 regime for 4 d followed by adjustment of  $OD_{750}$  to 0.1 and shifting to constant light. (C) Cells were grown under FL 50/500 regime for 4 d followed by adjustment of  $OD_{750}$  to 0.1 and shifting to constant light. (D) Cells were grown under FL 20/500 regime for 8 d followed by adjustment of  $OD_{750}$  to 0.1 and shifting to constant light. Mean  $\pm$  SD;  $n = 4$ . WT,  $\blacksquare$ ;  $\Delta flv1::flv1$  complementation strain,  $\star$ ; mutant strains  $\Delta flv1$ ,  $\circ$ ;  $\Delta flv3$ ,  $\nabla$ ;  $\Delta flv1/\Delta flv3$ ,  $\diamond$ .

cyanobacteria also harbor a mechanism to protect photosynthesis under such conditions. Likewise, the bioreactors that have so far been planned and tested for growth of cyanobacteria for biotechnology purposes are characterized by steep short-term fluctuations in light intensity upon rotation of the cells in bioreactors.

In contrast to the function of PGR5 as a safeguard of PSI in higher plants, we show that the PGR5-like protein in cyanobacteria is not important for survival under FL. Instead, we demonstrate an indispensable function of FDPs Flv1(A) and Flv3(A) in supporting photosynthesis and growth of cyanobacteria under FL conditions. The Flv1 and Flv3 proteins provide protection for sustainable function and protein stability of PSI upon abrupt high-light pulses by directing electrons to  $O_2$  in a harmless way without inducing damage in PSI.

## Results

**Growth Phenotype of Different Flv1 and Flv3 Mutants.** Flv1 and Flv3 proteins are ubiquitous in all cyanobacteria, yet no strong growth phenotype has been assigned for the mutants. Because the aquatic growth environments are typically characterized by fluctuations in the light intensity, we attempted to search for growth phenotypes in *Synechocystis*  $\Delta flv1$ ,  $\Delta flv3$ , and  $\Delta flv1/\Delta flv3$  mutants by exposing them to different intensities of constant light as well as to FL with low background illumination interrupted with repeating high-light pulses. *Synechocystis* mutants grown under constant low, normal-growth, and high-light conditions, 20, 50, and 500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , respectively, did not show any significant growth phenotype compared with wild type (WT; Fig. S1 A–C). In contrast, FL produced a strong growth phenotype in all these mutants. Under the FL 20/500 regime (20  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 5 min and 500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 30 s), the growth of the  $\Delta flv1$ ,  $\Delta flv3$ , and  $\Delta flv1/\Delta flv3$  mutant cells was completely inhibited (Fig. 1 A and B), whereas under FL 50/500 (50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 5 min and 500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 30 s) conditions, the mutant cells demonstrated drastic decrease of cell growth compared with WT (Fig. 1 A and C).

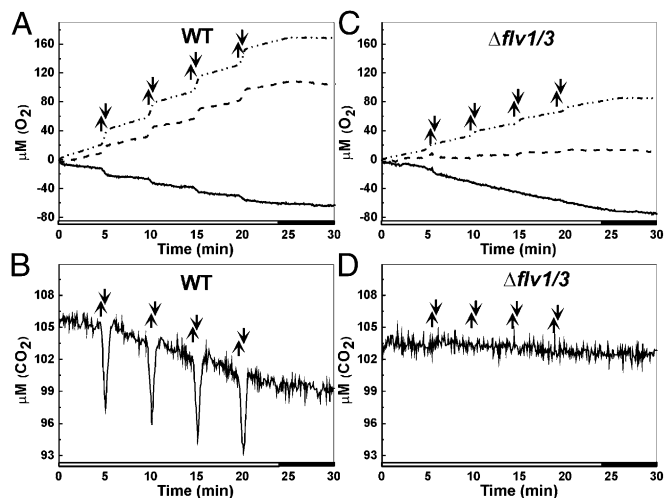
It was next examined whether the FL 20/500 and 50/500 regimes promote complete death of mutant cells or instead induce a growth arrest. To this end, the cells grown under two different FL regimes for 4 d were further diluted to  $OD_{750} = 0.1$  and

transferred to constant-light intensity of 50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Fig. 1 B and C shows that the mutant cells started to grow normally and reached nearly the same OD as WT cells after 4 d of growth at constant-light conditions. However, if the mutant cells were incubated under FL 20/500 regime for a longer period of time (8 d), the shift to constant light did not rescue the growth of the mutant cells (Fig. 1D). These results imply that mutant cells do not survive prolonged period of FL. Importantly, complementation of the *flv1* deletion with the functional Flv1 protein (the  $\Delta flv1::flv1$  strain) fully abolished the observed phenotype, confirming that the effect was indeed caused by the *flv1* deletion (Fig. 1B).

We likewise constructed the deletion mutants of Flv1A and Flv3A, the closest homologs of *Synechocystis* Flv1 and Flv3 proteins in filamentous *Anabaena* strains. Growth phenotypes of these *Anabaena* mutants (Fig. S1D), similar to those of the respective *Synechocystis* mutants, suggest a ubiquitous role for the Flv1 and Flv3 proteins in cyanobacteria in coping with FL conditions.

**Real-Time Gas-Exchange Analysis of the  $\Delta flv1/\Delta flv3$  Mutant.** To address the underlying molecular mechanisms for the cell growth arrest under FL in the absence of the Flv1 and Flv3 proteins, we next measured the photosynthetic gas exchange in the  $\Delta flv1/\Delta flv3$  mutant. Real-time gas-exchange analyses with membrane inlet mass spectrometry (MIMS) were performed with WT and mutant cells under light conditions mimicking the fluctuating growth conditions (20/500, four cycles then dark).

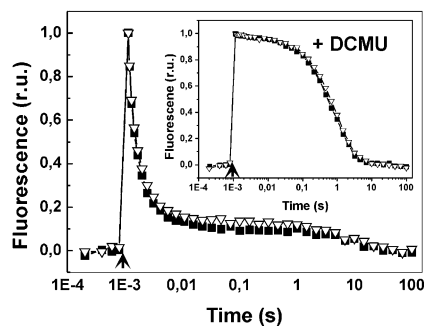
MIMS experiments with WT cells demonstrated, as expected, a strong signal of photosynthetic  $O_2$  evolution (Fig. 2A) and  $CO_2$  uptake (Fig. 2B) upon high-light pulses of FL, whereas the low-light phases repeatedly reduced the gas-exchange reactions to a significantly lower level. These signals showed conserved repetition in the amplitude of  $O_2$  and  $CO_2$  exchange from one high-light pulse to the next one (Fig. 2A and B).  $^{18}O_2$  labeling was used to discriminate the evolution and uptake of  $O_2$  during illumination of the cells. In WT, the  $O_2$  uptake upon low-light phases was equivalent or even lower (0.6  $\mu\text{mol } O_2\cdot\text{L}^{-1}\cdot\text{min}^{-1}$ ) than in the dark (0.7  $\mu\text{mol } O_2\cdot\text{L}^{-1}\cdot\text{min}^{-1}$ ). During high-light pulses, a strong  $O_2$  photoreduction was observed in WT cells (12.6



**Fig. 2.** Real-time  $O_2$  (A and C) and  $CO_2$  (B and D) exchange measurements in *Synechocystis* WT and  $\Delta flv1/\Delta flv3$  cells. The cells were grown under constant light of 50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and then set at  $OD_{750} = 0.4$ – $0.5$  and shifted to FL 20/500 for 2–3 d before the measurements. Actinic white light intensity of 20  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  was applied to the dark-adapted cells, and  $\sim 30$ -s high-light pulses at an intensity of 500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  was turned on (up arrows) and off (down arrows) every 4 min. Cumulative  $O_2$  evolution (dotted line),  $O_2$  uptake (solid line), and net photosynthesis (dashed line) were calculated from  $^{18}O_2/^{16}O_2$  exchange measurements.



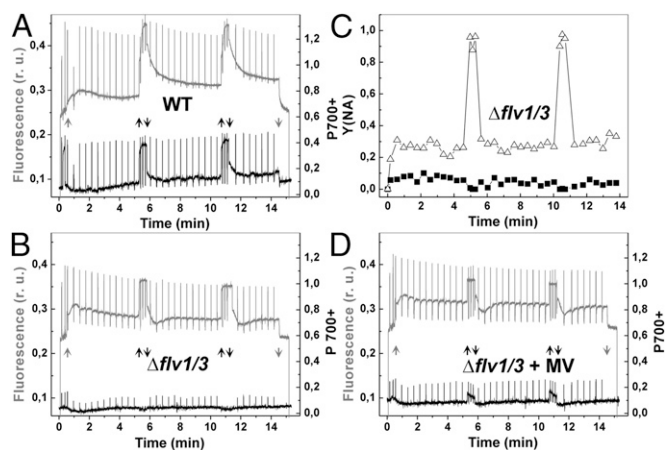




**Fig. 4.** Flash-induced increase in variable fluorescence and its subsequent decay in the WT and  $\Delta flv1/\Delta flv3$  cells in the absence and in the presence of DCMU (inset). WT (■) and  $\Delta flv1/\Delta flv3$  (▽) were grown under constant-light conditions of  $50 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , then set at  $\text{OD}_{750} = 0.4\text{--}0.5$  and shifted to FL 50/500 for 3 d before the measurements. r.u., relative units.

Incubation of the  $\Delta flv1/\Delta flv3$  mutants with methylviologen (MV), an artificial electron acceptor of PSI, resulted in only partial elimination of the acceptor side limitation of PSI, indicated by the appearance of the oxidized P700 fraction in the mutant cells upon a saturating light pulse fired during the high-light phase. MV also induced a small portion of open PSII centers during the high-light illumination phase of FL (Fig. 5D).

To reveal the molecular mechanism(s) behind such obscure modifications of the P700 oxidoreduction signals in the  $\Delta flv1/\Delta flv3$  mutant upon growth under FL, we next exposed the cells grown under constant-light conditions of  $50 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  to FL 50/500 and simultaneously recorded both the Chl fluorescence and the P700 oxidoreduction signals. During low-light phases of the FL experiment, the mutant cells grown under constant light did not show any distinct difference in the function of PSII or PSI compared with WT (Fig. 6A and B and Fig. S4A and B). The  $P_M$  values were also similar in the mutant ( $0.41 \pm 0.01$ ;  $n = 3$ ) and WT ( $0.40 \pm 0.02$ ;  $n = 3$ ) growing at constant-light conditions. During the subsequent low- to high-light transition, P700 became strongly oxidized in the WT cells (Fig. 6A and Fig. S4C). In contrast, only a very fast and transient oxidation of P700,



**Fig. 5.** PSII and PSI properties of the WT and  $\Delta flv1/\Delta flv3$  cells grown under FL. (A, B, and D) Chl fluorescence (gray line) and P700 (black line) were monitored simultaneously in WT (A) and  $\Delta flv1/\Delta flv3$  in the absence (B) and presence (D) of MV. (C) Acceptor-side limitation of PSI was calculated from WT (■) and  $\Delta flv1/\Delta flv3$  (△) in the absence of MV. The cells were grown under constant light, then set at  $\text{OD}_{750} = 0.4\text{--}0.5$  and shifted to FL 50/500 for 2–3 d before the measurements. Low background actinic light ( $58 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; gray arrow) and high light ( $530 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; black arrow) was switched on and off (up and down arrow, respectively), mimicking FL. r.u., relative units.

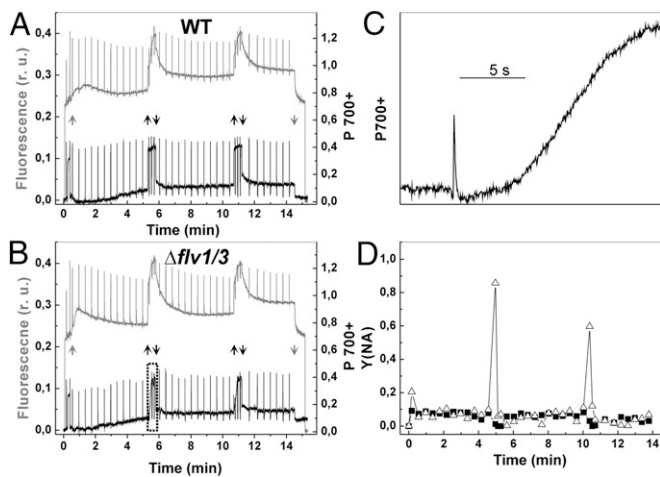
followed by prompt relaxation of  $P700^+$  occurred in the  $\Delta flv1/\Delta flv3$  mutant cells at the onset of the high-light phase of the FL cycles (Fig. 6B and C). After transient oxidoreduction, P700 remained completely reduced  $\sim 5$  s and reached a steady-state oxidation level only after  $\sim 15$  s of exposure to high light (Fig. 6C). Such a strong delay in P700 oxidation in mutants confirmed that the Flv1 and Flv3 proteins function as an important electron sink, and the absence of these proteins created strong and, importantly, repetitive acceptor-side limitation of PSI during the low- to high-light transitions of FL (Fig. 6D). Moreover, we monitored light-induced energization of membrane in WT and mutant cells treated with FL 50/500 for 3 d by using fluorescent pH indicator acridine yellow (AY). The mutant cells demonstrated approximately two times less light-induced changes in AY fluorescence (Fig. S5). Taking into account the fact that the relation between  $\Delta\text{pH}$  and changes in amine fluorescence is linear, we can assume that low  $\Delta\text{pH}$  values observed in the mutant might result in possible ATP starvation and consequently participate in causing the growth arrest in the  $\Delta flv1/\Delta flv3$  mutant cells under FL.

**Response of Photosynthetic Proteins to FL.** To clarify whether the distinct modification of PSI function under FL in the absence of the Flv1 and Flv3 proteins is reflected at the level of photosynthetic proteins, the photosynthetic apparatus was examined after 4 d of growth under FL using an immunoblotting approach and different protein-specific antibodies. The steady-state level of PsaB, the core protein of PSI, decreased  $>50\%$  in  $\Delta flv1$ ,  $\Delta flv3$ , and  $\Delta flv1/\Delta flv3$  mutant cells compared with WT, whereas D1, the PSII reaction center protein, was not significantly affected (Fig. 7A). No marked changes were observed in the amount of two different FNR isoforms and the Cyt f subunit of the *Cytb<sub>6</sub>f* complex. Instead, a significant down-regulation of RbcL, representing ribulose-1,5-biphosphate carboxylase/oxygenase, was evident in all studied mutant cells compared with WT. The  $\Delta flv1::flv1$  complementation mutant behaved similarly to WT in respect to photosynthetic proteins, but the Flv1 protein was highly up-regulated.

Next, we assessed dynamic changes in photosynthetic proteins by shifting WT and the  $\Delta flv1/\Delta flv3$  mutant from constant-growth light conditions to FL and by probing the total protein fraction with corresponding antibodies (Fig. 7B). In WT cells, the amount of PsaB started to increase after 3 d and was significantly up-regulated after 7 d of FL illumination. In contrast to this result, the  $\Delta flv1/\Delta flv3$  mutant cells demonstrated a drastic decrease of the PsaB protein on the third day of FL treatment, and the protein was nearly depleted after 7 d. The shift from constant-growth light to FL conditions did not affect the level of the D1 protein in WT. In the  $\Delta flv1/\Delta flv3$  mutant cells, the amount of the D1 protein was stable during the first 3 d of exposure to FL, but showed significant decrease on the seventh day. The protein amount of the RbcL started to decrease already from the first day of shift to FL in the  $\Delta flv1/\Delta flv3$  mutant cells. Furthermore, the shift from constant-growth light to FL 50/500 conditions for 7 d significantly increased protein carbonylation in the mutant cells, demonstrating high ROS production and strong oxidative damage to proteins (Fig. S6).

## Discussion

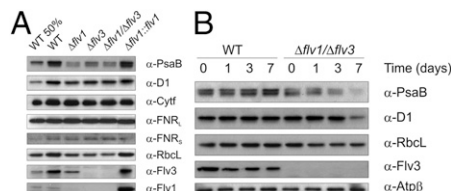
**Cyanobacteria and Plants Have Distinctly Different Strategies to Cope with FL.** Natural light environment of photosynthetic organisms is highly dynamic, which inflicts a threat to the function of the photosynthetic apparatus. Key regulatory mechanisms of higher plants to cope with rapidly fluctuating light intensities were recently addressed (8, 9). The natural light environment of cyanobacteria, typically the oceans and lakes, is even more challenging, with predictable fluctuations in light intensity as well as in the frequency of high-light pulses ( $<1$  Hz) far exceeding those occurring in the land. Indeed, a lens effect of waves produces excessively strong and also dim light, yet the intensity of light fluctuations decreases rapidly with increasing depth (12).



**Fig. 6.** PSII and PSI properties of the WT and  $\Delta flv1/\Delta flv3$  cells grown under constant light. (A and B) Chl fluorescence (gray line) and absorbance changes of P700 (black line) were recorded simultaneously in WT (A) and  $\Delta flv1/\Delta flv3$  (B). (C) Oxidation of 700 during low- to high-light transitions in  $\Delta flv1/\Delta flv3$ . (D) Acceptor-side limitation of PSI in WT (■) and  $\Delta flv1/\Delta flv3$  (△). The cells were grown under constant light, then set at  $OD_{750} = 0.4-0.5$  and shifted to FL 50/500 for 2–3 d before the measurements. Low-background actinic light ( $58 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; gray arrow) and high light ( $530 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; black arrow) were switched on and off (up and down arrow, respectively), mimicking FL. r.u., relative units.

To elucidate cyanobacterial strategies to cope with FL, we tested whether one of the most important components involved in safeguarding the photosynthetic apparatus of higher plants under FL, the PGR5 protein, has a similar function in cyanobacteria. This hypothesis, however, turned out not to be the case. Indeed, deletion of the *pgr5* homolog from the *Synechocystis* genome did not render the cells susceptible to FL (Fig. S1E), which contradicts with the behavior of the *pgr5* mutant of *Arabidopsis* (9). Other proteins and mechanisms, so far identified as important for higher plants to cope with FL environments—for example, the STN7 kinase (8)—do not exist in cyanobacteria because of completely different architecture of the light harvesting systems.

Because mechanisms enabling plants to cope with abrupt fluctuations in light intensity are not present or functional in cyanobacteria, it is highly conceivable that the respective cyanobacterial mechanisms are not conserved in higher plants. Considering the possible cyanobacterial strategies to cope with FL, we addressed the role of FDPs, a unique family of cyanobacterial proteins that are involved in electron transfer processes. Two of the cyanobacterial FDPs (Flv2 and Flv4) function



**Fig. 7.** Immunoblots demonstrating the relative amounts of proteins in the WT;  $\Delta flv1$ ,  $\Delta flv3$ , and  $\Delta flv1/\Delta flv3$  mutants; and complementation strain. (A) Protein content of the WT and mutant strains grown 4 d under FL 50/500 regime was analyzed with corresponding antibodies. (B) Changes in the protein content of WT and the  $\Delta flv1/\Delta flv3$  mutant upon shift from constant-growth light (day 0) to FL 50/500 for 1, 3, and 7 d. Proteins from total cell extracts ( $10 \mu\text{g}$  in each well) were separated by SDS/PAGE, and immunoblotting was performed by using specific antibodies. Flv1 and Flv3 proteins were detected from soluble samples ( $30 \mu\text{g}$  in each well).

in photoprotection of PSII (2, 3), whereas the Flv1 and Flv3 proteins in *Synechocystis* function in accepting electrons after PSI and directing them to  $\text{O}_2$  in the so-called “Mehler-like” reaction that does not produce ROS (4, 5). Corresponding proteins in filamentous *Anabaena* cells are called Flv1A and Flv3A (7).

**Flv1 and Flv3 Proteins Enable Growth and Photosynthesis of Cyanobacteria Under FL Conditions.** Despite earlier research showing the function of Flv1 and Flv3 as a light-induced  $\text{O}_2$  uptake mechanism (4, 5), attempts to find any environmental condition that would induce detrimental growth phenotype for the  $\Delta flv1$  and/or  $\Delta flv3$  mutants compared with WT *Synechocystis* have so far failed. Only the  $\Delta flv3$  mutant has occasionally been shown to have a reduced capability to cope with high light (6).

Intriguingly, the exposure of the  $\Delta flv1$  (A) and/or  $\Delta flv3$  (A) mutants of *Synechocystis* and *Anabaena* to FL clearly demonstrated the indispensable role of these FDPs in survival and growth of cyanobacterial cells under conditions of abrupt changes in light intensity. Subjecting the cells to two different background illuminations, but keeping the intensity and frequency of high-light pulses constant, revealed that the background light intensity determines the severity of FL stress (Fig. 1 B and C). It is conceivable that the Flv1 and Flv3 proteins coevolved in cyanobacteria together with oxygenic photosynthesis to protect the photosynthetic apparatus upon strong fluctuations in light intensity characteristic to aquatic environments.

**PSI Is the Primary Target of FL in the Absence of the Flv1/Flv3 Heterodimer.**

The importance of the Flv1 and Flv3 proteins as an electron sink becomes evident only under FL—i.e., when the background low light is repeatedly interrupted with high-light pulses. More detailed analysis of the photosynthetic apparatus revealed that the function of PSI, but not of PSII, was strictly compromised in the  $\Delta flv1/\Delta flv3$  mutant (Figs. 4 and 5). For further inspection of reasons behind such a severe phenotype, the  $\Delta flv1/\Delta flv3$  mutant cells were grown under constant light and then subjected to actinic FL during measurements with DUAL-PAM. This study demonstrated a delay in oxidation of P700 upon the low- to high-light transition (Fig. 6C), due to strong acceptor-side limitation of PSI. In the thylakoid membrane of plants, strong PSI acceptor-side limitation results in leaking of electrons to  $\text{O}_2$ , thus generating superoxide radicals not only at the stromal side, but also within the thylakoid membrane, most likely originating from the  $A_1$  electron acceptor under conditions when the Fe-S centers are reduced (13). Enhanced back-reactions due to the acceptor-side limitation of PSI also have a capacity to result in Chl triplet formation and consequently in formation of singlet oxygen that is known to be extremely hazardous for the protein environment (14). Cyanobacteria possess the Flv1 and Flv3 proteins, which are functioning as a strong and efficient electron sink during overreduction of electron transport chain by preventing the acceptor-side limitation of PSI and thus also ROS production. The  $\Delta flv1$  and  $\Delta flv3$  mutant cells grown under constant light do not show acceptor-side limitation of PSI during steady-state low light (Fig. 6D). Conversely, if the mutants were grown under constant light and shifted to FL conditions, the extreme and, importantly, repeated acceptor-side limitation of PSI, was observed during the low- to high-light transition phases (Fig. 6D), resulting in the strong and recurrent overreduction of electron-transport chain. At earlier stages of the FL treatment (up to 3 d), production of ROS and oxidative damage to proteins were not significant (Fig. S6), but at relatively lower levels, ROS might also function in triggering a signaling cascade to down-regulate the expression of PSI proteins. Indeed, a marked loss of PSI proteins was detected during 3 d of the FL treatment, whereas no changes in PSII proteins were observed (Fig. 7B). Upon prolonged FL treatment, a preferential loss of PSI complexes led to an increase in PSII-to-PSI ratio in the  $\Delta flv1/\Delta flv3$  mutant. During low background light of FL, PSI could still cope with the relatively small amount of electrons coming from PSII. Upon abrupt high-light pulses, however, the overreduction of the

intersystem electron-transfer chain kept P700 reduced, thus completely blocking linear electron flow and inhibiting photosynthesis. An impairment of net photosynthesis in the  $\Delta flv1/\Delta flv3$  mutants, which implies decreased CO<sub>2</sub> fixation, is in line with observed down-regulation of PSI complexes and RbcL subunits of RubisCO under FL. After prolonged incubation under FL, malfunction of PSI and, consequently, strongly increased ROS production and oxidative stress led to the irreversible cell damage and death (Fig. S6). Indeed, the Flv1 and Flv3 proteins in cyanobacteria are crucial to maintaining the redox balance of the electron-transfer chain, which provides protection for PSI under FL.

Finally, the complementation strain, by revealing the WT characteristics of growth and reverting the functional status of the photosynthetic apparatus, confirmed that the presence of the Flv1/Flv3 heterodimer provides cyanobacterial cells with a harmless way to dissipate excess electrons from PSI, which in turn is essential to sustaining photosynthesis under FL conditions (Figs. 1B and 7 and Table 1).

### Concluding Remarks

We have demonstrated here an indispensable role of the Flv1 and Flv3 proteins in safeguarding of PSI and the survival of cyanobacteria under FL. Another FDP heterodimer, Flv2/Flv4, was previously shown to be crucial for photoprotection of PSII under high light and/or Ci-deprivation in  $\beta$ -cyanobacteria. Indeed, cyanobacteria, the progenitors of higher plant chloroplasts, have unique mechanisms to protect the photosynthetic apparatus against ROS. Intriguingly, the phylogenetic tree of the FDPs reveals gradual disappearance of FDPs upon evolution of higher plants. We postulate that the development of oxygenic photosynthesis in cyanobacteria was accompanied by evolution of the unique FDPs that provided a safe function of the photosynthetic machinery under oxygenic environment. Gradual disappearance of FDPs during evolution is apparently related to their futile function in storage of reducing equivalents, and they were gradually replaced by more “energy-efficient” mechanisms to regulate photosynthetic electron flow. It is conceivable that the movement of life from oceans to the land made such evolution possible.

### Materials and Methods

**Strains and Culture Conditions.** *Synechocystis* and *Anabaena* WT and the mutant strains were grown in BG-11 medium buffered with 20 mM TES *N*-

Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-KOH (pH 8.2) under continuous light (50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 30 °C and 3% (vol/vol) CO<sub>2</sub>. Experiments were performed in growth chambers (AlgaeTron AG 130-ECO; PSI) equipped with LED white light, and the cells were shifted to air-level CO<sub>2</sub>. The following light regimes were applied: constant light of 20, 50, and 500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (dim, normal, and high light, respectively); FL 20/500 (20  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 5 min/500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 30 s); and FL 50/500 (50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 5 min/500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 30 s). For all physiological experiments, the cells were first grown under constant light of 50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , then inoculated to OD<sub>750</sub> = 0.4–0.5 and shifted to FL 20/500 or 50/500 for 3–4 d before the measurements. For activity measurements, the cells were harvested and resuspended in a fresh BG-11 medium at Chl concentration of 10 or 15  $\mu\text{g}\cdot\text{mL}^{-1}$ .

**Mutant Strains.** The  $\Delta flv1$  and  $\Delta flv3$  single and  $\Delta flv1/\Delta flv3$  double mutants have been described (4, 5). The construction of the  $\Delta flv1::flv1$  complementation strain, *pgr5* deletion mutant in *Synechocystis*, and *flv1A* and *flv3A* deletion mutants of *Anabaena* are described in *SI Materials and Methods* and Fig. S7.

**MIMS.** Measurements of <sup>16</sup>O<sub>2</sub> (mass 32), <sup>18</sup>O<sub>2</sub> (mass 36), and CO<sub>2</sub> (mass 44) exchange were performed simultaneously by MIMS directly from suspension cultures as described (ref. 5; *SI Materials and Methods*).

**Fluorescence and P700 Measurement.** Chl fluorescence and P700 oxidation were recorded by DUAL-PAM-100 (Walz). Mimicking of FL during measurements was performed by changing the intensity of applied red actinic light (635 nm) between 58 and 530  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

**Protein Isolation, Electrophoresis, and Immunodetection.** Total cell extract and the soluble fraction of *Synechocystis* cells were isolated as described in ref. 2. Proteins were separated by 12% SDS/PAGE containing 6 M urea. The proteins were electrotransferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore) and immunodetected by protein-specific antibodies. Additional information is provided in *SI Materials and Methods*.

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