Functional redundancy between flavodiiron proteins and NDH-1 in *Synechocystis* sp. PCC 6803

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SUMMARY

In oxygenic photosynthetic organisms, excluding angiosperms, flavodiiron proteins (FDPs) catalyze light-dependent reduction of O2 to H2O. This alleviates electron pressure on the photosynthetic apparatus and protects it from photodamage. In *Synechocystis* sp. PCC 6803, four FDP isoforms function as hetero-oligomers of Flv1 and Flv3 and/or Flv2 and Flv4. An alternative electron transport pathway mediated by the NAD(P)H dehydrogenase-like complex (NDH-1) also contributes to redox hemostasis and the photoprotection of photosynthesis. Four NDH-1 types have been characterized in cyanobacteria: NDH-11, and NDH-12, which function in respiration; and NDH-13 and NDH-14, which function in CO2 uptake. All four types are involved in cyclic electron transport. Along with single FDP mutants (Δflv1 and Δflv3) and the double NDH-1 mutants (Δd1d2, which is deficient in NDH-11,2 and Δd3d4, which is deficient in NDH-13,4), we studied triple mutants lacking one of Flv1 or Flv3, and NDH-11,2 or NDH-13,4. We show that the presence of either Flv1/3 or NDH-1,2, but not NDH-1,3,4, is indispensable for survival during changes in growth conditions from high CO2/moderate light to low CO2/high light. Our results show functional redundancy between FDPs and NDH-1,2 under the studied conditions. We suggest that ferredoxin probably functions as a primary electron donor to both Flv1/3 and NDH-1,2, allowing their functions to be dynamically coordinated for efficient oxidation of photosystem I and for photoprotection under variable CO2 and light availability.

Keywords: Flavodiiron proteins, Flv, NDH-1, photosynthesis, *Synechocystis* sp. PCC 6803, cyanobacteria, Mehler-like reaction, alternative electron transfer, photoprotection

INTRODUCTION

Photosynthetic organisms have evolved a variety of different regulatory mechanisms, which are important for the protection of the photosynthetic machinery during rapid changes in environmental conditions. Fluctuating light intensities present particular risk to the photosystems due to over-reduction of the photosynthetic electron transport chain (PETC), particularly photosystem (PS) I (Allahverdiyeva et al., 2015; Tiwari et al., 2016; Shimakawa et al., 2016). To counter this, cyanobacteria, algae and plants (excluding angiosperms) employ C-type flavodiiron proteins (FDPs) as a strong photoprotective electron sink, directing excess photosynthetic electrons from downstream of PSI to O2 (Helman et al., 2003; Allahverdiyeva et al., 2013; Gerotto et al., 2016; Ilik et al., 2017; Chaux et al., 2017; Jokel et al., 2018; Alboresi et al., 2019). This process is referred to as the Mehler-like reaction and, in contrast to the Mehler reaction (Mehler, 1957), does not produce harmful reactive oxygen species (Vicente et al., 2002; Brown et al., 2019). The genome of the β-cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) encodes four isoforms of FDPs, namely Flv1-4, which function in O2 photoreduction mainly as hetero-oligomers consisting of either Flv1 and Flv3 or Flv2 and Flv4 (Zhang et al., 2004; Mustila et al., 2016; Santana-Sanchez et al., 2019). Recently, we showed that under air level [CO2], Flv1/3 and Flv2/4 hetero-oligomers function in a coordinated and interdependent manner: Flv1/3 stimulates strong but transient O2 photoreduction upon the onset of illumination or during increases in light intensity, while Flv2/4 hetero-oligomers catalyze a slower and
limited steady-state reduction of $O_2$, also using electrons downstream of PSI (Santana-Sanchez et al., 2019). In elevated $[CO_2]$ and in air $[CO_2]$ at alkaline pH 9, the flv4-flv2 operon encoding Flv2, Flv4 and the SII0218 protein is down-regulated (Zhang et al., 2009; Santana-Sanchez et al., 2019). Nevertheless, a low level of Flv1/3 in elevated $[CO_2]$ stimulates strong steady-state $O_2$ photooxidation, whereas in air $[CO_2]$ at pH 9, Flv1/3 is solely responsible for strong but transient $O_2$ photoreduction (Santana-Sanchez et al., 2019). Whilst \textit{in vitro} assays showed that homo-oligomers of recombinant Flv1, Flv3 and Flv4 can reduce $O_2$ with NADH and/or NADPH (Vicente et al., 2002; Shimakawa et al., 2015; Brown et al., 2019), ferredoxin-NADP$^-$-reductase (FNR) and reduced ferredoxin (Fd) are yet to be considered as possible donors to FDPs. Moreover, the use of \textit{Synechocystis} mutants solely overexpressing Flv1 or Flv3 clearly demonstrated that, in contrast to \textit{in vitro} experiments, homo-oligomers of Flv3 or Flv1 are not involved in $O_2$ photoreduction \textit{in vivo} (Mustila et al., 2016). Thus, the electron donor to FDPs remains to be elucidated \textit{in vivo}.

In cyanobacteria, photosynthetic and respiratory electron transfer reactions coexist on thylakoid membranes and are intricately linked, necessitating strict regulation (Mullineaux, 2014). The NAD(P)H dehydrogenase-like complex 1 (NDH-1, photosynthetic complex I) is mainly localized in thylakoid membranes and involved in several bioenergetic reactions, including cyclic electron transfer (CET) around PSI, respiration and $CO_2$ acquisition via the carbon concentrating mechanism (CCM) (for reviews see Battchikova et al., 2011; Ma and Ogawa, 2015; Burnap et al., 2015; Peltier et al., 2016). The NDH-1 complex is present as several forms with distinct physiological roles: the NDH-1$^\Delta_1$ type features the subunits NdhD1 and NdhF1 and functions as complex I of the respiratory electron transfer chain (Zhang et al., 2004; He et al., 2015; Saura and Kaila, 2019); the NDH-1$^\Delta_2$ form features the NdhD2 subunit instead of NdhD1, but it remains unclear whether NDH-1$^\Delta_1$ and NDH-1$^\Delta_2$ serve different physiological functions (Peltier et al., 2016). The NDH-1$^\Delta_3$ and NDH-1$^\Delta_4$ forms that comprise low-Ci-inducible, high-affinity $CO_2$ acquisition subunits NdhD3, NdhF3, CupA and CupS, or constitutive, low-affinity $CO_2$ acquisition subunits NdhD4, NdhF4 and CupB, respectively, function in conversion of $CO_2$ to $HCO_3^-$ (Ohkawa et al., 2000a,b; Shibata et al., 2001; Zhang et al., 2004; Burnap et al., 2013; Han et al., 2017; Schuller et al., 2019). This process is possibly enabled by alkeine pockets at the thylakoid membrane created by NDH-1 proton pumping (Kaplan and Reinhold, 1999). All four NDH-1$^\Delta_4$ types catalyze CET from Fd to plastocquinone (PQ), which is coupled to the pumping of 4$H^+$/2$e^-$ into the thylakoid lumen (He et al., 2015; Laughlin et al., 2019; Schuller et al., 2019; Saura and Kaila, 2019). However, CET mediated by NDH-1$^\Delta_3$ is minor in comparison to CET mediated by NDH-1$^\Delta_2$ (Bernat et al., 2011).

In the current study, we aim to elucidate how the electron transport pathways mediated by FDPs and NDH-1 cooperate to allow the maintenance of redox poise between the PETC, respiration, CCM and $CO_2$ fixation in the Calvin–Benson–Bassham cycle (CBB) under variable light and $C_i$ availability. To this end, we employed both biophysical and biochemical methods to characterize various \textit{Synechocystis} mutant strains with combined deficiencies of both FDP and NDH-1 pathways. Our results provide convincing evidence for the presence of either Flv1/3 or NDH-1$^\Delta_1$, but not NDH-1$^\Delta_3$, being indispensable for the survival of \textit{Synechocystis} cells under transitions from high $[CO_2]$ conditions to the combined stress conditions of air $[CO_2]$ and high light. We show that the dynamically coordinated and cooperative function of Flv1/3 and NDH-1$^\Delta_1$ is required for the photoprotection of the photosynthetic apparatus of \textit{Synechocystis} cells and discuss the molecular mechanisms involved. We suggest that this coordination is enabled by a shared electron donor, as Fd probably functions as the main reductant of both Flv1/3 and NDH-1$^\Delta_1$, although the possible contribution of NADPH as a donor cannot be fully excluded.

RESULTS

Simultaneous inactivation of Flv1/3 and NDH-1$^\Delta_1$ is lethal upon shift from high to air $[CO_2]$ and high light

To examine whether the simultaneous inactivation of NDH-1 and FDPs has an adverse effect on cell survival, we monitored the growth of \textit{Synechocystis} wild-type (WT) and various mutant strains lacking (i) either Flv1 or Flv3 ($\Delta$flv1 and $\Delta$flv3, respectively; Helman et al., 2003), (ii) both NdhD1 and NdhD2 ($\Delta$ndh12, deficient in NDH-1$^\Delta_1$ and NDH-1$^\Delta_2$; Ohkawa et al., 2000b), (iii) both NdhD3 and NdhD4 ($\Delta$ndh34, deficient in NDH-1$^\Delta_3$ and NDH-1$^\Delta_4$; Ohkawa et al., 2000b), as well as triple mutants with combined deficiencies of both pathways ($\Delta$flv1 $\Delta$d1d2, $\Delta$flv3 $\Delta$d1d2, $\Delta$flv1 $\Delta$d3d4, $\Delta$flv3 $\Delta$d3d4) in conditions of differing $CO_2$ availability and light intensity. The triple mutants exhibited similar or slightly slower growth compared with the WT and other mutant strains under high $[CO_2]$ (3% $CO_2$) and moderate light (ML, intensity of 220 $\mu$mol photons m$^{-2}$ sec$^{-1}$) (Figure 1a). We also detected no substantial difference in growth when the 3% $CO_2$/ML pre-grown cells were diluted (optical density (OD)$_{750}$ = 0.1) and subjected simultaneously to air $[CO_2]$ and high light stress conditions (HL, intensity of 220 $\mu$mol photons m$^{-2}$ sec$^{-1}$), the growth of both $\Delta$flv3 $\Delta$d1d2 and $\Delta$flv1 $\Delta$d1d2 strains was completely inhibited (Figure 1d). Importantly, $\Delta$flv1, $\Delta$flv3 and $\Delta$d1d2 strains demonstrated growth similar to WT cells under air $[CO_2]$/HL conditions.

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We next examined whether pre-adaptation to air [CO\(_2\)] would rescue the growth arrest. When cells previously adapted to air [CO\(_2\)]/ML were diluted (OD\(_{750}\) = 0.1) and shifted to air [CO\(_2\)]/HL, no differences in growth patterns between the strains were detected (Figure 1c). Importantly, the triple mutants lacking either Flv1 or Flv3 and NDH-1,2,3,4 (Δflv1 d3d4 and Δflv3 d3d4 strains) did not show a lethal phenotype after a shift from 3% [CO\(_2\)]/ML to air [CO\(_2\)]/HL, although somewhat reduced growth was observed compared with the control strains (Figure S1b,d). This suggests that, under the studied conditions, cooperation of Flv1/3 with NDH-1,2,3,4 complexes is not as crucial as with NDH-1,2.

Our attempts to create M55/Δflv1 and M55/Δflv3 double mutants lacking either Flv1 or Flv3 and the central membrane component of the NDH-1 complex, NdhB, were both unsuccessful. We were unable to obtain any M55/Δflv1 colonies and the M55/Δflv3 mutant strain could not be segregated (Figure S1e), suggesting that the absence of both Flv1/3 and NDH-1 disrupts essential cell metabolism.

We also studied the growth of the mutant strains on agar plates containing BG-11 (Figure 1e). For these experiments, 3% CO\(_2\)/ML-grown cells were diluted and grown on plates under either air [CO\(_2\)]/ML or air [CO\(_2\)]/HL conditions for 7 days. The Δflv1 and Δflv3 and Δd1d2 mutants did not exhibit any visible differences in growth capacity under these conditions in comparison with the WT. In contrast, the growth of the Δflv1 d1d2 and Δflv3 d1d2 triple mutants was strongly reduced under air [CO\(_2\)]/ML, while no growth was detected under air [CO\(_2\)]/HL (Figure 1e). The Δflv1 d3d4 and Δflv3 d3d4 triple mutants demonstrated a slow growth phenotype, similarly to that previously reported for Δd3d4 on agar plates (Ohkawa et al., 2000b).

As the growth phenotypes of Δflv1 and Δflv3, Δflv1 d1d2 and Δflv3 d1d2 were similar under all conditions, we only included Δflv3 and Δflv3 d1d2 in the majority of subsequent experiments.

Either Flv1/3 or NDH-1,2,3 is required for the oxidation of PSI during sudden increases in light intensity

The lethal phenotype observed upon the shift to air [CO\(_2\)]/HL, which was caused by the simultaneous impairment of PSI.
the Mehler-like reaction catalyzed by Flv1/3 (Helman et al., 2003; Allahverdiyeva et al., 2011, 2013) and by NDH-1,2 complex-mediated respiration and CET (Ohkawa et al., 2000b; Bernat et al., 2011) suggests a functional redundancy between these electron transport pathways. To investigate this possibility further, we used membrane inlet mass spectrometry (MIMS) to examine the kinetics of O$_2$ and CO$_2$ exchange upon HL illumination of dark-adapted WT and mutant cells grown for 4 days in air [CO$_2$/ML]. To distinguish O$_2$ uptake from photosynthetic gross O$_2$ evolution under illumination, we enriched cell suspensions with $^{16}$O$_2$ before the measurements. In dark-adapted WT cells, a transient peak in O$_2$ uptake occurs during the first minute of illumination (Figure 2a). We showed recently that this transient peak is attributed to the Mehler-like reaction catalyzed predominantly by Flv1/3 hetero-oligomers, while Flv2/4 hetero-oligomers mainly contribute to steady-state light-induced O$_2$ reduction in an interdependent manner (Sanchez-Sanchez et al., 2019). In $\Delta$flv3 cells, light-induced O$_2$ reduction was almost abolished and only a slight impairment of the rate of photosynthetic gross O$_2$ evolution was observed in comparison with WT cells (Figure 2b). This result is in agreement with previous studies (Helman et al., 2003; Allahverdiyeva et al., 2013; Santana-Sanchez et al., 2019).

In contrast to the WT, $\Delta$d1d2 cells lacked a fast decay phase of light-induced O$_2$ uptake during the first minute of illumination, demonstrating sustained O$_2$ photoreduction at high levels [approximately 75–100 $\mu$mol O$_2$ mg chlorophyll (Chl)$^{-1}$ h$^{-1}$] throughout the illumination period. Meanwhile, gross O$_2$ evolution was diminished nearly two-fold compared with the WT (Figure 2c). Thus, sustained O$_2$ photoreduction in $\Delta$d1d2 is not due to increased electron flow from PSI, but most probably due to increased activity of FDPs. Indeed, $\Delta$flv3 d1d2 mutant cells showed only minor light-induced O$_2$ uptake transiently during the first minute of illumination (Figure 2d). The $\Delta$flv3 d1d2 cells demonstrated slower induction of photosynthetic O$_2$ evolution following two-component kinetics, and slightly impaired steady-state gross O$_2$ evolution compared with WT (Figure 2d).

The initial peak in CO$_2$ uptake rate at the onset of illumination probably reflects activation of the CCM (Liran et al., 2018). Accordingly, this initial peak is absent in the M55 mutant deficient in NDH-1,4 in addition to NDH-1,2, as well as in WT cells grown at pH 6 (Figure S2). No salient impairment of CCM was observed in any of the studied strains under air [CO$_2$/ML, with CO$_2$ uptake rates peaking at approximately 1.5 mm CO$_2$ mg Chl$^{-1}$ h$^{-1}$. In WT as well as in $\Delta$flv3, CO$_2$ fixation is then induced and the respiratory compensation point surpassed after approximately 1 min of illumination (Figure 2a,b). However, in $\Delta$d1d2, induction of CO$_2$ fixation is severely delayed and the cells only reached the compensation point at the end of the 5-min illumination period (Figure 2c). Intriguingly, although a slight delay was observed also in $\Delta$flv3 d1d2, induction of CO$_2$ fixation was largely recovered in the triple mutant (Figure 2d).

These MIMS results indicated that combined deficiency of Flv1/3 and NDH-1,2 under standard growth conditions, air [CO$_2$/ML, slightly perturbs photosynthetic electron transfer and the redox poise between the PETC and cytosolic sink reactions. Next, we examined the ability of the mutant strains to adjust their photosynthetic activity to variable light conditions by measuring Chl a fluorescence simultaneously with P700 redox changes in conditions where light intensity periodically fluctuated between low (25 $\mu$mol photons m$^{-2}$ sec$^{-1}$, LL) and high irradiance (530 $\mu$mol photons m$^{-2}$ sec$^{-1}$, HLI). Cells lacking Flv3 suffered from transient PSI acceptor side limitation Y(NA) upon sudden increases in light intensity, resulting in delayed oxidation of PSI (Figure 3a), which is in line with

**Figure 2.** O$_2$ and CO$_2$ exchange rates in wild-type and mutant strains. Cells were grown in air [CO$_2$/moderate light for 4 days, after which the cells were harvested and chlorophyll (Chl) a concentration adjusted to 10 $\mu$g ml$^{-1}$ with fresh BG-11. Cells were dark-adapted for 15 min, and gas exchange was monitored by membrane inlet mass spectrometry over a 5-min illumination period with 500 $\mu$mol photons m$^{-2}$ sec$^{-1}$ of white actinic light. Before the measurements, samples were supplemented with $^{16}$O$_2$ at an equivalent concentration to $^{18}$O$_2$ to distinguish O$_2$ uptake from O$_2$ evolution, and with 1.5 mM NaHCO$_3$. Dashed line in each panel indicates the compensation point of CO$_2$ fixation (uptake rate equals respiratory rate). The experiment was repeated with three independent biological replicates, of which representative measurements are shown.
The effective yield of PSII was slightly decreased in HL in the HL phases in the LL phases of the experiment (Figure 3a), suggesting a slightly elevated acceptor-side limitation during subsequent cycles. This implies a compensatory mechanism that is distinct from Flv1/3 hetero-oligomers, such as, for example, NDH-1 mediated electron transport.

We detected no acceptor-side limitation during the HL phases in Δd1d2 (Figure 3a) as PSI was oxidized similarly to the WT (Figure 3b). However, interestingly, Δd1d2 cells exhibited slightly elevated acceptor-side limitation during the LL phases of the experiment (Figure 3a), suggesting a role for NDH-1,2 in maintaining photosynthetic redox poise in light-limited conditions. In the triple mutant strain Δflv3 d1d2, transitions from LL to HL as well as from dark to light caused severe limitation on the acceptor side of PSI, resulting in an inability to oxidize PSI during periodic 1-min HL illumination. Unlike the Δflv3 mutant, Δflv3 d1d2 did not show improvement in the PSI acceptor side limitation during subsequent cycles. This implies a compensating activity of NDH-1 in Δflv3 mutant under the studied conditions. Interestingly, diminished donor side limitation in Δflv3 d1d2 resulted in slightly elevated effective yield of PSI during the HL phases (Figure 3c). This could indicate the attenuation of pH-dependent limitation of electron transfer at Cyt b5f, due to impairment of proton motive force (pmf) generation in CET and the Mehler-like reaction. The effective yield of PSII was slightly decreased in HL in Δflv3 and Δd1d2, but not in Δflv3 d1d2 (Figure 3d).

The differences in photosynthetic electron transport reported above may also be contingent on altered redox states of the NADP+/NADPH pool. Therefore, to investigate the effect of Flv3 and/or NDH-1,2 deficiency on NADP+/NADPH redox kinetics, we recorded NADPH fluorescence changes from dark-adapted cells simultaneously with Chl a fluorescence in conditions where actinic light intensity fluctuated between 25 (LL) and 530 μmol photons m−2 sec−1 (HL) similarly to Figure 3. Upon dark-to-LL transitions, NADPH rapidly accumulated close to a maximal amount in WT, Δflv3 and Δd1d2 cells, while in Δflv3 d1d2 the NADPH pool was highly reduced already in darkness. Reoxidation of NADPH then occurred in WT and Δflv3 d1d2 cells, while in Δflv3 a slower oxidation phase preceded a transient re-reduction phase during the first minute of illumination (Figure 4a,b). Strong reduction of the NADP+ pool was also detected in Δflv3 during the second HL phase of the experiment. In Δd1d2 cells, very little oxidation of the NADPH pool occurred during illumination, even at HL–LL transitions (Figure 4c). Chl a fluorescence also remained at an elevated level (Figure 4c), suggesting a reduced PQ pool. This was possibly due to delayed activation of CO2 fixation in the CBB cycle (Figure 2c), and impaired CET or dark respiration. In contrast, NADPH was strongly oxidized in Δflv3 d1d2 cells at HL–LL transitions (Figure 4d), but was predominantly reduced in dark-adapted cells (Figure 4d). Upon cessation of illumination, oxidation of the NADPH pool was followed by transient re-reduction after approximately 10 sec. As observed previously (Schreiber and Klughammer, 2009; Holland et al., 2015), the re-reduction peak coincided with a secondary post-illumination rise in Chl a fluorescence (PIFR), while oxidation of NADPH paralleled an initial PIFR. The post-illumination re-reduction of NADP+ was diminished and both Chl a PIFR peaks were missing in Δd1d2 (Figure 4c). Therefore, the PIFR peaks
Figure 4. NADPH and chlorophyll (Chl) a fluorescence under fluctuating light in wild-type (WT) and mutant strains. (a) WT, (b) Δflv3, (c) Δd1d2, and (d) Δflv3 d1d2 cells were grown in air [CO2]/moderate light for 4 days, after which the cells were harvested and Chl a concentration adjusted to 5 μg ml⁻¹ with fresh BG-11. Cells were dark-adapted for 20 min, and then subjected to illumination with red actinic light alternating between 25 and 530 μmol photons m⁻² sec⁻¹ in 1-min periods. NADPH redox changes were monitored with the Dual-PAM 100 spectrophotometer and its 9-AA/NADPH accessory module by measuring fluorescence changes between 420 and 580 nm induced by excitation at 365 nm, as described by (Schreiber and Klughammer, 2009) and (Kauny and Setif, 2014). Chl a fluorescence was recorded simultaneously. (e) NADPH redox changes during illumination of dark-adapted cells with 530 μmol photons m⁻² sec⁻¹. Other experimental details are as in a–d. (f) Shows a magnification of the first 2 sec of illumination in (e). Values are normalized according to minimum and maximum fluorescence changes at the onset and cessation of illumination, respectively. Experiments were repeated with three independent biological replicates, of which representative measurements are shown. HL, high light/irradiance; LL, low light/irradiance; r.u., relative units.

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probably originate from cyclic and respiratory electron transport via NDH-1,2 (Holland et al., 2015). However, both Chl PIFR peaks were observed in Δflv3 d1d2 cells, indicating involvement of a complementary pathway, possibly via succinate dehydrogenase (Cooley and Vermaas, 2001), or a CET pathway dependent on PGR5 and PGR1-like (Yermenko et al., 2005; Dann and Leister, 2019). Finally, we monitored NADPH redox kinetics under conditions mimicking the MIMS experiments, where strong O2 photoreduction occurs in WT and Δd1d2 during the first minute of dark-to-high light transition (Figure 2). No substantial differences were observed between the strains, as close to maximal reduction of NADP+ was obtained within approximately 0.25 sec (Figure 4f) and maintained throughout a 40-sec illumination period (Figure 4e). This argues against NADPH being a primary electron donor to either Flv1/3 or NDH-1,2, at least during fast time scales, although based on the current data, we cannot exclude the possibility of NADPH contribution during longer time scales or in steady-state conditions.

**Redox status of PSI donor and acceptor side electron carriers and build-up of pmf during dark-to-light transitions depend on both Flv1/3 and NDH-1,2**

To elucidate the molecular mechanism behind the photosynthetic phenotypes of the studied mutant strains, we next utilized a DUAL-KLAS-NIR spectrophotometer to distinguish between redox changes of plastocyanin (PC), P700 and Fd (Klughammer and Schreiber, 2016; Schreiber, 2017; Setif et al., 2019) upon exposure of dark-adapted cells to HL (503 μmol photons m−2 sec−1). Upon illumination of WT cells, rapid oxidation of P700 and PC occurred, followed by transient reduction of PC, P700, as well as Fd after approximately 0.2 sec (Figure 5a). Reoxidation of all three electron carriers then ensued after approximately 0.5 sec. In Δflv3 cells, P700 and PC remained mostly reduced after the initial oxidation transient until approximately 3 sec, when another transient and partial reoxidation peak of P700 and PC occurred at approximately 8 sec (Figure 5b). This was followed by re-reduction of P700, similar to that observed recently by Bulychev et al. (2018). After 15 sec in light, P700 and PC became gradually oxidized. In contrast to WT, Fd remained reduced for several seconds in light, and was only slowly reoxidized over the 30 sec illumination period (Figure 5b). These observations indicate the importance of Flv1/3 as an electron sink to O2, accepting electrons presumably from reduced Fd (after approximately 0.5 sec in light). In Δd1d2, redox changes of PC, P700 and Fd were similar to WT, except that the reduction of P700- following initial oxidation as well as reduction of Fd occurred already after approximately 50 msec (Figure 5c). In Δflv3 d1d2, re-reduction of P700- and reduction of Fd also occurred already after approximately 50 msec (Figure 5d), suggesting involvement of NDH-1,2 as an acceptor of electrons from Fd at that stage. Fd was reduced more quickly in Δflv3 d1d2 than in any other strains and was slowly reoxidized over 30 sec (Figure 5d), probably due to the shortage of electron acceptors (Figure 3a). In summary, these NIR-spectroscopic measurements revealed that Flv1/3 and NDH-1,2 control the redox poise between PSI, PC and primary electron acceptor Fd during specific time frames at transitions to HL. The results are congruent with Fd functioning as the electron donor to both NDH-1,2 and Flv1/3.

To inspect whether the phenotypes observed above depend on differential build-up or regulation of pmf, we measured in vivo changes in the pmf by monitoring the absorbance change difference between 500 and 480 nm, which constitutes the electrochromic shift (ECS) in Synechocystis (Viola et al., 2019). In vivo measurement of light-induced ECS revealed that after 1 sec of illumination of dark-adapted WT cells with 500 μmol photons m−2 sec−1, high pmf level was transiently generated, followed by decline during the subsequent seconds (Figure 5e). After approximately 10 sec of illumination, pmf again increased towards a steadier value (Figure 5e). Congruently with the strong reduction of P700 and Fd (Figure 5b), the initial pmf peak after the first second of illumination was heavily dependent on the presence of Flv1/3. In both Δflv3 and Δflv3 d1d2, pmf and thylakoid proton flux (vH+1) were lower than in WT after 1 sec (Figure 5g). In Δflv3, pmf remained drastically lower than in WT during the first seconds of illumination, but differed only slightly from WT thereafter due to diminished conductivity of the thylakoid membrane (gH+) (Figure 5f), which is mainly determined by the activity of the adenosine triphosphate (ATP) synthase (Cruz et al., 2005; Viola et al., 2019). In Δd1d2, the thylakoid proton flux was similar to WT (Figure 5g), probably due to enhanced FDP activity (Figure 2c) compensating for impaired CET and respiration. However, pmf remained lower than in WT throughout the 1-min experiment (Figure 5e) because of elevated conductivity (Figure 5f). In contrast, despite drastically lower proton flux (Figure 5g), Δflv3 d1d2 cells maintained pmf close to the WT levels after the first seconds in light (Figure 5e) due to diminished conductivity (Figure 5f). This indicates that the increased acceptor side limitation of PSI in Δflv3 d1d2 in comparison with Δflv3 (Figure 3a) is not caused by lack of photosynthetic control as a consequence of lowered pmf generation in CET. Based on the comparison of ECS kinetics in single, double and triple mutant cells studied here, we conclude that both Flv1/3 and NDH-1,2 contribute to proton flux during transitions from dark to HL. However, deficiency in NDH-1,2 in Δd1d2 is mostly compensated by elevated FDP activity in terms of proton flux, while ATP synthase activity is increased, possibly as a response to the delay in activation of carbon fixation (Figure 2c). In contrast, impaired thylakoid proton flux in Δflv3 during
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(a) WT vs. PC, P700, Fd in Δflv3, Δd1d2, Δflv3 d1d2

(b) Relative redox change (%max NIR ΔI/I)

(c) ECS (∆I/I 500-480 nm)

(d) ECS (∆I/I 500-480 nm)

(e) ECS vs. Time (s)

(f) gH⁺ vs. Time (s)

(g) VH⁺ vs. Time (s)

(h) ECS vs. Time (s)

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dark-to-light transitions cannot be compensated by NDH-1. Instead, downregulation of ATP-synthase activity lowers thylakoid conductivity and allows maintenance of pmf (apart from the first seconds of illumination) in Δflv3 and, even more dramatically, in Δflv3 d1d2.

**PSI content is diminished in triple mutants deficient in Flv1/3 and NDH-1,2 shifted to air [CO2] and HL**

The results from the growth assays, along with the real-time gas exchange, P700 redox change and ECS measurements suggested that in Δd1d2 cells, an increase in the activity of FDPs compensates for the absence of a functional NDH-1,2 complex, allowing efficient oxidation of PSI under HL. Conversely, in Δflv1 and Δflv3 mutants, over-reduction of the electron transport chain occurs initially upon exposure to HL, but another mechanism(s) eventually allow acclimation and survival in high [CO2] and high light (Helman et al., 2003). Under air [CO2] Flv1/3, hetero-oligomers catalyze transient O2 photoreduction, which is why no strong growth phenotype is observed in Δflv1 or Δflv3 under constant HL (Allahverdiyeva et al., 2013; Santana-Sanchez et al., 2019). However, when both Flv1/3 and NDH-1,2 are absent and the electron sink capacity of the cytosol is not elevated by high [CO2], cells are unable to oxidize PSI in high light, possibly resulting in lethal photodamage to PSI. To assess this hypothesis, we employed immunoblotting to provide estimates of the protein content of PSI (D1) and PSI (PsaB) subunits, orange carotenoid protein (OCP), large isoform of the Fd-NADP+ oxidoreductase (FNRL), FDPs, as well as NdhD3 and the bicarbonate transporter SbtA after 24 h exposure to different growth conditions.

WT and Δflv1 or Δflv3 cells grown at 3% [CO2]/ML and shifted to air [CO2]/ML at OD = 0.1, accumulated similar levels of the PSI reaction center protein D1 and the PSI subunit PsaB. However, we detected a moderate decrease in the amount of both D1 and PsaB in the Δflv1 d1d2 and Δflv3 d1d2 triple mutants, while an increased amount of both proteins was detected in Δd1d2 (Figure 6a). After 24 h exposure to air [CO2]/HL, PsaB amount had decreased even further in Δflv1 d1d2 and Δflv3 d1d2 (Figure 6c). Furthermore, 77K fluorescence spectra revealed that while Δflv3 d1d2 and WT cells grown under air [CO2]/ML had similar PSI/PSII ratios (Figure 6d), the 24-h exposure to air [CO2]/HL caused a dramatic decrease in the relative PSI fluorescence cross-section (Figure 6e). This strongly supports the hypothesis that loss of PSI contributes to the lethality of the shift to air [CO2]/HL. Interestingly, a substantial decrease in D1 content in air [CO2]/HL was also observed in the triple mutants (Figure 6b,c).

Importantly, the Δflv1 d1d2 and Δflv3 d1d2 strains were unable to induce substantial accumulation of the proteins encoded by the flv4-2 operon after a shift from 3% to air [CO2] (Figure 6a,c). Only when pre-grown for 4 days in air [CO2]/ML before being shifted to higher irradiance, the expression of the operon was induced (Figure 6b). Similar to flv4-2 operon proteins, both NdhD3 and SbtA failed to accumulate after shifts from 3% to air [CO2]/HL in the triple mutants, which probably impairs CCM and contributes to the lethal phenotype of the triple mutants in those conditions. Only small amounts of NdhD3 and SbtA were detected also in triple mutant cells shifted from 3% [CO2] to air [CO2]/ML (Figure 6a). However, while closer to the WT amount of NdhD3, only a small amount of SbtA was detected after 24 h in air [CO2]/HL (shifted from air [CO2]/ML) (Figure 6b). In the Δd1d2 mutant, the NdhD3 content was similar to WT, but Flv3 as well as the flv4-2 operon proteins (Flv2, Flv4 and Sl0218) were upregulated in all conditions tested (Figure 6), which probably contributes to the increased rate of O2 photoreduction in that strain (Figure 2c). The amounts of OCP and of FNRL were unchanged in the mutant strains in all conditions (Figure 6).

**DISCUSSION**

Flv1/3 hetero-oligomers have been shown to be essential for photoreduction of O2 as a rapid response to excessive reduction of PSI, and consequently, for cell survival under...
fluctuating light conditions (Allahverdiyeva et al., 2013; Santana-Sanchez et al., 2019). Moreover, we recently showed that in air [CO2], Flv2/4 hetero-oligomers also catalyze low-level light-induced steady-state reduction of O2 on the acceptor side of PSI in a coordinated manner with Flv1/3 (Santana-Sanchez et al., 2019).

Another alternative electron transport component, the NDH-1 complex, in turn, functions in CET around PSI, using Fd as the electron donor (Saura and Kaila, 2019; Schuller et al., 2019). Besides CET, the NDH-1 complexes, incorporating the NdhD1 or NdhD2 subunit, are involved in respiration, whereas NDH-1 complexes, incorporating the NdhD3 or NdhD4 subunit, function in CO2 uptake. In the current study, we have used Synechocystis mutant strains with different combinations of deficiencies in Flv1/3 and NDH-1 to examine the

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physiological significance of these alternative electron transport pathways and the possibility of functional interdependence between them. Our results provide compelling evidence for the concomitant physiological functions of Flv1/3 and NDH-1,2 and their dynamic coordination for the efficient oxidation of PSI (thus protecting it from photodamage) under variable light conditions, and when low [CO2] limits the consumption of reductants in the CBB cycle. The two pathways can compensate for each other's absence to some extent, but absence of both the Flv1/3 hetero-oligomer and NDH-1,2 is lethal when cells are transferred from elevated [CO2] to the combined condition of air level [CO2] and HL (Figure 1d,e). In addition to the absence of these two important pathways, lethality is due to an inability to accumulate low C3-inducible photoprotective CCM proteins (Figure 6c). Combined deficiency of Flv1/3 and NDH-1,4 did not result in a lethal phenotype upon similar shifts, indicating that functional redundancy exists specifically between the Mehler-like reaction and NDH-1,2.

**Coordinated functions of Flv1/3 and NDH-1,2 protect PSI by maintaining redox poise between the PETC and carbon fixation**

Cells lacking functional Flv1/3 hetero-oligomers suffer from transient over-reduction of PSI during sudden increases in light intensity (Figures 3 and 5) due to the impairment of the Mehler-like reaction (Figure 2; Allahverdiyeva et al., 2013). Whilst NAD(P)H has been proposed as the electron donor to Flv3 (Vicente et al., 2002; Brown et al., 2019) and Flv1 homo-oligomers (Brown et al., 2019), in vivo experiments have been unsupportive (Mustila et al., 2016), with exact electron donor(s) to FDP hetero-oligomers yet to be proven. *In vivo* experiments performed in this study demonstrate that the quick reoxidation of Fd after 0.5 sec (Figure 5a) in light is absent in the Δflv3 deletion strains, whereby Fd remains strongly reduced (Figure 5b). This result, together with the lack of impairment in NADPH-reduction and oxidation kinetics of Δflv3 deletion strains (similar conditions, Figure 4f) provide strong support for Fd, rather than NAD(P)H, being the primary electron donor to the Flv1/3 hetero-oligomer *in vivo*. Accordingly, Fd has been shown to interact with Flv1 and Flv3 by Fd chromatography (Hanke et al., 2011) and with Flv3 using a two-hybrid assay (Cassier-Chauvat and Chauvat, 2014).

The deficiency of NDH-1,2, in turn, reduced the ability of cells to maintain oxidized P700 and Fd under HL soon after (approximately 50-200 msec) a dark-to-light transition (Figure 5c). This brief time scale may be due to PSI-NDH-1 supercomplexes (Gao et al., 2016) where P700 can be oxidized rapidly upon the onset of illumination. It has been shown that formation of the NDH-1-PSI supercomplex is important to keep PSI functional under various stress conditions (Zhao et al., 2017). NDH-1,2 deficiency also caused slightly elevated acceptor side limitation of PSI under LL (Figure 3a), which was probably due to a lack of NADPH oxidation during the low light phases of fluctuating light (Figure 4c). Thus, NDH-1,2 appears to play an important role under low light, as has been previously suggested for chloroplastic NDH in angiosperms (Yamori et al., 2011; Yamori et al., 2015) and bryophytes (Ueda et al., 2012). The delayed activation of CBB in Δd1d2 did not result in an inability to oxidize PSI (Figures 3b and 5c), probably due to significant enhancement of FDP-mediated O2 photoreduction (Figure 2c) providing an enlarged electron sink for the PETC. An opposite order of causation is also possible, whereby the excessive funneling of photosynthetic electrons to O2 would cause the delay in induction of CO2 fixation in Δd1d2. However, the observation that there is no NADPH shortage at the onset of illumination in Δd1d2, and rather the consumption of NADPH during transitions from HL to LL is impaired (Figure 4c), suggests that the availability of reductant for the CBB cycle is not the limiting factor. Nor is it probably CO2, as CCM is functioning as in WT (Figure 2c), or ATP, as ATP synthase activity was even higher than in WT in Δd1d2 (Figure 5f). One possibility is that the increased funneling of electrons from Fd to the Mehler-like reaction in Δd1d2 (Figure 2c), while not impairing FNR function (and thus NADPH production), might cause a shortage of electrons for the ferredoxin-thioredoxin reductase. This would impair light-dependent activation of the CBB cycle by the thioredoxin system (Michelet et al., 2013; Guo et al., 2014). In addition, it might explain the delay observed in CO2 fixation in Δd1d2. Nevertheless, simultaneous deficiency of FDPs in addition to NDH-1,2 (in Δflv3 d1d2 triple mutant), with the effect of diminishing the flow of photosynthetic electrons to O2 photoreduction, mostly rescued the delay in CO2 fixation (Figure 2d) as well as NADPH consumption (Figure 4d) seen in Δd1d2. Transient O2 photoreduction at a low rate was still observed in Δflv3 d1d2 during dark-to-LL transitions (Figure 2d), possibly mediated by the thylakoid terminal oxidases (Ermakova et al., 2016) or by photorespiration (Allahverdiyeva et al., 2011). However, the triple mutants have more severe inability to oxidize PSI than Δflv3 during sudden increases in irradiance (Figure 3). These observations suggest that, in addition, Flv1/3 and NDH-1,2 have a role in contributing to oxidation of PSI during changes in light conditions or carbon availability. Upon deficiency of NDH-1,2 (in Δd1d2), cells prioritize the protection of PSI over efficient CO2 fixation by upregulating the Mehler-like reaction via an unknown mechanism. The triple mutants cannot do this, leading to the timely induction of CO2 fixation at the high cost of inability to oxidize PSI (Figure 3a) or Fd (Figure 5d). This results in loss of PSI (Figure 6), possibly due to photodamage to its FeS clusters (Tiwari et al., 2016; Shimakawa et al., 2016).
On the mechanism of NDH-1-mediated oxidation of PSI

The contribution of NDH-1 to oxidation of PSI during sudden increases in light intensity is not unprecedented. In angiosperms, where FDPs have been lost during evolution (Ilik et al., 2017), Arabidopsis mutants lacking the NDH complex show a delay in oxidation of PSI during increases in light intensity in comparison with Arabidopsis WT (Nikkane et al., 2018; Shimakawa and Miyake, 2018a). By definition, canonical CET cannot directly increase the relative proportion of oxidized P700, as electrons from the acceptor side of PSI are shunted back to the intersystem electron transfer chain. However, NDH-1 may enhance PSI oxidation by at least three alternative, but mutually non-exclusive mechanisms.

i NDH-1-mediated CET is coupled to the translocation of protons from cytosol to the thylakoid lumen with a 2H+/e⁻ stoichiometry (Strand et al., 2017; Saura and Kaila, 2019). Therefore, it will contribute to build-up of ΔpH, which limits electron transfer to PSI by inhibiting PQH₂ oxidation at Cyt b6f (Shimakawa and Miyake, 2018b) and drives ATP synthesis to accommodate the needs of the CBB, increasing its electron sink capacity. As the Mehler-like reaction also contributes to build-up of ΔpH by consuming H⁺ on the cytosolic side of the thylakoid membrane and by supporting linear electron flow (Figure 5), (Allahverdiyeva et al., 2013), enhanced FDP activity in Δd1d2 (Figure 2c) partly compensates for the lack of NDH-1,2 in respect to the generation of proton flux (Figure 5g). However, this fails to explain the exacerbated impairment of P700 and Fd oxidation in Δflv3 d1d2 in comparison with Δflv3 (Figures 3 and 5) because in the triple mutant pmf was not lower than in Δflv3 (Figure 5e). However, adequate pmf is maintained at the expense of ATP synthase activity (Figure 5f), and it is likely that diminished ATP production contributes to the increased acceptor side limitation of PSI in Δflv3 d1d2 (Figure 3a). Therefore, NDH-1,2 could contribute to P700 oxidation by enhancing cytosolic sink capacity by providing a more suitable ATP/NADPH ratio for the CBB. Adjustment of the ATP/NADPH ratio closer to the theoretically optimal 3:2 has long been considered a fundamental reason for the existence of CET (Kramer et al., 2004; Yamori and Shikanai, 2016).

ii NDH-1-mediated respiratory electron transfer, that is, coupling of PQ reduction by NDH-1 to O₂ reduction by thylakoid terminal oxidases (i.e. Cyt and Cox), would also contribute to oxidation of PSI by relieving electron pressure in the intersystem chain during illumination (Ernakova et al., 2016), as well as contribute further to ΔpH by consuming H⁺ on the cytosolic side of thylakoids (Cyd) and pumping protons to the lumen (Cox) (Brändén et al., 2006). Interestingly, Liu et al. (2012) have shown that the subcellular localization of NDH-1 complexes is dependent on the redox state of the PQ pool. An oxidized PQ pool causes NDH-1 to accumulate at specific clusters in thylakoid membranes where it would probably transfer electrons (via the PQ pool) to a terminal oxidase. A reduced PQ pool, in turn, results in a more even distribution of NDH-1 within thylakoids (Liu et al., 2012). However, it is important to note that terminal oxidases do not have a high electron sink capacity (Ernakova et al., 2016).

iii NDH-1 has been predicted, albeit not yet experimentally shown in photosynthetic organisms, to be able to function in reverse: to oxidize PQH₂ driven by concomitant release of protons from the thylakoid lumen (Strand et al., 2017). Such reverse activity would constitute a “pseudo-linear” electron transfer pathway that would bypass PSI and thereby prevent its over-reduction. This could occur in conditions where the PQ pool is reduced, pmf is high and the Fd pool is oxidized (Strand et al., 2017). Such conditions probably exist transiently during dark-to-light and LL-to-HL transitions (Figures 4 and 5) (Strand et al., 2019). Accordingly, fast re-reduction of P700⁺ already after 50 msec was observed in Δd1d2 and Δflv3 d1d2 during dark-to-HL transitions (Figure 5e). As supported by the impaired ability to oxidize Fd in the absence of Flv1/3 (Figure 5), the presence of the Flv1/3-catalyzed Mehler-like reaction would probably be essential in this model to maintain the Fd pool in a sufficiently oxidized state to provide electron acceptors for reverse-functioning NDH-1. However, it is noteworthy that NDH-1 reverse activity would also have the effect of lowering ΔpH, thereby relieving photosynthetic control at Cyt b6f. This could counteract the effect of any reverse NDH-1 activity by increasing electron flow to PSI.

Hypothetical mechanisms for the coordination of Flv1/3 and NDH-1,2 activities are shown in Figure 7.

Inability to induce a strong CCM network contributes to lethality upon shifts to air [CO₂]/HL in cells deficient in both Flv1/3 and NDH-1,2

Exposure of Synechocystis cells to low [CO₂] induces expression of high-affinity CCM-related genes such as SbtA, a HCO₃⁻/Na⁺ symporter on the plasma membrane, and NdhD3, which is part of the NDH-1 complex on thylakoids specializing in C₅ uptake (Okawa et al., 2000b; Shibata et al., 2001; Shibata et al., 2002; Zhang et al., 2004). CCM is energetically expensive, but the large C₅ flux involved in the operation of CCM contributes to dissipation of excess light energy under stress conditions (Xu et al., 2008; Burnap et al., 2015). NDH-1,2 in particular has a photoprotective role, using reduced Fd to drive CO₂ conversion to HCO₃⁻ with concomitant translocation of protons into the thylakoid lumen.
Despite the low induction level of the CCM proteins, NdhD3 and SbtA, the triple mutants (Figure 6) survive under standard growth conditions of air [CO₂]_ML (Figure 1b,c), but their growth was retarded on solid media (Figure 1e). The difference may be due to the differential diffusivity of CO₂ in solid media in comparison with suspension cultures (Ohkawa et al., 2000a); that is, the impairment of CCM in the triple mutants becomes more limiting on solid media. However, when a decrease in [CO₂] is coupled with an increase in irradiance a low amount of CCM, reflected by the low accumulation level of NdhD3 and SbtA in the ∆flv1dl2d and ∆flv3dl2d triple mutants (Figure 6c) fails to dissipate excess energy, thus resulting in photodamage and lethality of those conditions. The regulatory mechanisms controlling the inability of the triple mutants to accumulate low Ci-inducible proteins are unclear and remain to be elucidated. Accumulation of NADP⁺ and α-ketoglutarate inhibits the induction of CCM gene expression via interaction with the transcription factor NdhR (CcmR) (Daley et al., 2012). NdhR also controls expression of the Flv4-2 operon encoding Flv2, Flv4 and SII0218 (Eisenhub et al., 2012), all of which also showed decreased accumulation levels in the triple mutants (Figure 6). However, at least in the tested conditions, no substantial increase in the relative amount of NADP⁺ was detected in ∆flv3dl2d cells (Figure 4). Nevertheless, it is important to note that Flv1/3 and NDH-1,3,4 cooperation alone is not crucial for cell metabolism, as the ∆flv1 d3d4 and ∆flv3 d3d4 mutant cells do not show clear growth phenotypes (Figure S1).

Conclusions

In the current study, we have shown that FDPs and NDH-1 function cooperatively to maintain redox balance between the PETC and cytosolic carbon assimilation upon sudden changes in light intensity and/or carbon availability. It is probable that both pathways receive electrons primarily from Fd, which enables coordinated regulation of their activities. Cooperation of FDPs and NDH-1 has an essential photoprotective role during shifts to air [CO₂]HL, contributing to efficient oxidation of PSI, build-up of pmf and induction of expression of low Cᵢ-specific CCM-related genes. A key issue yet to be elucidated is the regulatory mechanism(s) by which the dynamic coordination of FDP and NDH-1 activities is achieved. One potential regulatory mechanism could function through the light-dependent Fd-thioredoxin system, which by competing with FDPs and NDH-1 for electrons from Fd, could constitute an effective regulatory feedback-loop. Indeed, light-dependent redox-sensitive cysteine residues have been identified in Flv1 and Flv3 as well as in several subunits of NDH-1 (Guo et al., 2014), and activity of chloroplastic NDH-1 in plants has been reported to be regulated by thioredoxins (Courteille et al., 2013; Nikkanen et al., 2018).

Finally, it is worth noting that simultaneous removal of Flv1/3 and NDH-1,1,2 as competitive electron sinks downstream of PSI may be a useful biotechnological tool for maximizing the direction of photosynthetic electrons to desired pathways under controlled environmental conditions (McCormick et al., 2013; Thiel et al., 2019; Jokel et al.,...
2019). Furthermore, our findings about the functional interplay between FDPs and NDH-1 will be highly relevant in projects aiming to enhance crop productivity via introduction of exogenous FDPs to higher plants (Yamamoto et al., 2016; Gómez et al., 2018).

**EXPERIMENTAL PROCEDURES**

**Strains and culture conditions**

In the current study we used the glucose-tolerant WT strain *Synechocystis* sp. PCC 6803 (Williams, 1988), single mutant strains Δflv1 and Δflv3 (Helman et al., 2003), M55 mutant (ΔndhB) (Ogawa, 1991), double mutants Δd1d2 and Δd3d4 (Ohkawa et al., 2000a) and triple mutants Δflv1 d1d2, Δflv3 d1d2, Δflv1 d3d4 and Δflv3 d3d4 obtained from the CyanoMutants collection (Nakamura et al., 1999). The triple mutants were constructed by T. Ogawa in Δd1d2 and Δd3d4 backgrounds. All the mutants demonstrated complete segregation in the presence of antibiotic. Pre-experimental cultures were always grown in 30 ml batches of BG-11 medium complete segregation in the presence of antibiotic. Pre-experimental cultures were grown in 30 ml batches of BG-11 medium pH 7.5 (Williams, 1988) under 3% [CO2] a t30°C under continuous white light of 50 µmol photons m–2 sec–1 (ML) with agitation. Mutant pre-cultures were supplemented with the appropriate antibiotics. At the logarithmic growth phase, cells were harvested and resuspended in fresh BG-11 without antibiotics at OD750 of 0.1–0.2, as described in the appropriate figure legends. Cells were then shifted to air [CO2]30°C and illuminated continuously with white light of either 50 or 220 µmol photons m–2 sec–1, as described in the figure legends.

**Gas exchange measurements**

The exchange of 16O2 (m/z 32), 18O2 (m/z 36) and CO2 (m/z 44) was measured in vivo with MIMS as described in Mustila et al. (2016). Harvested cells were resuspended in fresh BG-11 pH 7.5 and adjusted to 10 μg Chl a ml–1, and kept for 1 h in air [CO2]50 µmol photons m–2 sec–1. Before measurements, cells were supplemented with 18O2 at an equivalent concentration to 16O2 and with 1.5 mM NaHCO3. Cells were dark-adapted for 15 min, after which gas exchange was monitored over a 5-min illumination period of 500 µmol photons m–2 sec–1 of white actinic light (AL). The gas exchange rates were calculated according to Beckmann et al. (2008).

**Measurement of fluorescence and absorbance changes**

Experimental cultures for all spectroscopic experiments were grown for 4 days in air [CO2]50 µmol photons m–2 sec–1 at 30°C in BG-11 pH 7.5. Chl a fluorescence and P700 oxido-reduction (875–930 nm absorbance difference) were simultaneously recorded (Figure 3) with the Dual-PAM 100 spectrophotometer (Walz, Effeltrich, Germany). Harvested cells were resuspended in fresh BG-11 pH 7.5 and adjusted to 10 μg Chl a ml–1, and kept for 1 h in air [CO2]50 µmol photons m–2 sec–1 dark-adapted for 10 min before being subjected to a fluctuating light regime alternating between 1-min periods of 25 and 530 µmol photons m–2 sec–1 of red AL. Saturating pulses (500 msec of 5000 µmol photons m–2 sec–1) were administered at 15 sec intervals. Photosynthetic parameters were calculated as follows: Y(NA) = (Fm – Fm′)/Fm; Y(ND) = Pm/Fm; Y(I) = 1 – Y(ND) – Y(NA) (Klughammer and Schreiber, 2008); Y1 (II) = (Fm′ – Fm)/Fm′ (Genty et al., 1989).

A DUAL-KLAS-NIR spectrophotometer (Walz) was used to measure absorbance difference changes at 780–820, 820–870, 840–965 and 870–965 nm, from which PC, P700 and Fd redox changes (Figure 5) were deconvoluted based on differential model plots (DMPs) for PC, P700 and Fd (Klughammer and Schreiber, 2016; Schreiber, 2017; Setif et al., 2019). The redox changes were then normalized according to the maximal redox changes determined with the NIRMAX script of the instrument software (Figure 3b), consisting of a 3 sec illumination of red AL (200 µmol photons m–2 sec–1), with a saturating pulse after 200 msec of illumination to reduce the Fd pool fully. After 4 sec of darkness thereafter, cells were illuminated under far red light for 10 sec with a saturating pulse administered in the end of the illumination period to oxidize P700 fully. In WT Synechocystis, very fast reoxidation of Fd upon illumination impedes the measurement of model spectra. To circumvent this issue, we measured the DMPs from Δflv3 d1d2 cells where P700 oxidation was severely delayed in comparison with WT (Figure 3b). The DMPs (Figure S3a) were measured using scripts provided with the instrument software. Harvested cells were adjusted to 10 μg Chl a ml–1 with fresh BG-11 pH 7.5, and kept for 1 h in air [CO2]50 µmol photons m–2 sec–1 and dark-adapted for 30 min before illumination for 30 sec with 503 µmol photons m–2 sec–1 of red AL. It is noteworthy that cytochrome c3 and (Fm,Fd) redox changes probably contribute to the PC and Fd signals, respectively (Setif et al., 2019).

NADPH fluorescence changes between 420 and 580 nm, induced by excitation at 365 nm, were measured simultaneously with Chl a fluorescence with the Dual-PAM 100 and its 9- AA/NADPH accessory module (Walz) (Schreiber and Klughammer, 2005; Kauny and Setif, 2014). Experimental samples were prepared as above and [Chl a] was adjusted to 5 µg ml–1. Cells were dark-adapted for 20 min and subjected to the same fluctuating light regime as in Figure 3, but without saturating pulses. The ECS was measured as the absorbance difference between 500 and 480 nm (Viola et al., 2019). Absorbance changes were measured from dark-adapted cell suspensions with a JTS-10 spectrophotometer (BioLogic, Seyssinet-Pariset, France) using 500 and 480 nm CWL 50 10 nm FWHM bandpass filters (Edmund Optics, Barrington, NJ, USA) and BG39 filters (Schott, Mainz, Germany) protecting the light detectors from scattering effects. Absorbance changes induced by measuring light only were subtracted from changes under ML + AL. ECS values were normalized to ECS change induced by a single turnover flash provided by an XST-103 xenon lamp (Walz). Experimental samples were prepared as above and [Chl a] was adjusted to 7.5 µg ml–1, and illuminated with green AL of 500 µmol photons m–2 sec–1 for 1 or 60 sec interspersed with 500 msec dark intervals at 2, 7.5, 18, 30 and 45 sec. Pm/f was calculated as the extent of ECS decrease at the dark intervals. Thylakoid conductivity (gh) was calculated as the inverse of the time constant of a first-order fit to ECS relaxation kinetics during a dark interval, and proton flux (vH+) as pmf × gh+ (Cruz et al., 2005). Representative ECS traces of whole 60 sec measurements (upper panels) as well as fast kinetics during dark intervals after 30 sec illumination (lower panels) from WT, Δflv3, Δd1d2 and Δflv3 d1d2 cells are shown in Figure 5h.

77K fluorescence emission spectra were measured with a QE Pro spectrophotometer (Ocean Optics, Dunedin, FL, USA). Cells were harvested and [Chl a] was adjusted to 7.5 µg ml–1 with fresh BG-11 pH 7.5. Cells were frozen in liquid N2 and excited at 440 nm. Raw spectra were normalized to the PSII-fluorescence peak at 685 nm.

**Protein extraction and immunoblotting**

Total protein extracts from cultures were isolated as previously described (Zhang et al., 2009), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels with 6 wurea
and blotted on polyvinylidene fluoride membranes. Membranes were probed with antibodies raised against D1, PsaB (AS10 695; Agrisera, Vännäs, Sweden), PetH (kindly shared by H. Matthijs), OCP (kindly shared by D. Kirilovsky), SbtA (kindly shared by T. Ogawa), NdhD3 (Eurogentec, Liège, Belgium), Flv2, Flv4 and Flv3 (Antiprot, Puchheim, Germany), and SIlo218 (MedProbe, Oslo, Norway). Horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Chicago, IL, USA) and Amersham ECL (GE Healthcare) were used for detection.

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AUTHOR CONTRIBUTIONS

LN and YA conceptualized the research; LN, ASS, ME and YA designed the research; ME and ASS conducted growth experiments; LN performed MIMS and spectrophotometric experiments; ASS performed immunoblotting; all authors analyzed the data; LN and YA wrote the article with input from ASS and ME.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and its Supporting information.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Growth of Δflv1 d3d4 and Δflv3 d3d4 and creation of the M55-Δflv3 mutant strains.

Figure S2. O2 and CO2 fluxes in M55 and in WT at pH 6.

Figure S3. Near-infrared (NIR) differential model plots (DMPs) for deconvolution of PC, P700 and Fd signals with the DUAL-KLAS-NIR 100 spectrometer.

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