Title: Flv3A facilitates O₂ photoreduction and affects H₂ photoproduction independently of Flv1A in diazotrophic *Anabaena* filaments

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Running title: The role of vegetative-cell specific Flv1A and Flv3A in *Anabaena*

Summary

The model heterocyst-forming filamentous cyanobacterium, *Anabaena* sp. PCC 7120 (*Anabaena*) represents multicellular organisms capable of simultaneously performing oxygenic photosynthesis in vegetative cells and the O₂-sensitive N₂-fixation inside the heterocysts. The flavodiiron proteins (FDPs) have been shown to participate in photoprotection of photosynthesis by driving excess electrons to O₂ (Mehler-like reaction). Here, we addressed the physiological relevance of the vegetative cell-specific Flv1A and Flv3A on the bioenergetic processes occurring in diazotrophic *Anabaena* under variable CO₂. We demonstrate that both Flv1A and Flv3A are required for proper induction of the Mehler-like reaction upon a sudden increase in light intensity, which is likely important for the activation of carbon-concentrating mechanisms (CCM) and CO₂ fixation. Under ambient CO₂ diazotrophic conditions, Flv3A is capable of mediating moderate O₂ photoreduction, independently of Flv1A, but in coordination with Flv2 and Flv4. Strikingly, the lack of Flv3A resulted in strong downregulation of the heterocyst-specific uptake hydrogenase, which led to enhanced H₂ photoproduction under both oxic and micro-oxic conditions. These results reveal a novel regulatory network between the Mehler-like reaction and the diazotrophic metabolism, which is of great interest for future biotechnological applications.

52 Introduction

53 Filamentous heterocyst-forming cyanobacteria such as *Anabaena* sp. PCC 7120 (hereafter
54 *Anabaena*) represent a unique group of prokaryotes capable of simultaneously performing two
55 conflicting metabolic processes: (i) O₂-producing photosynthesis in vegetative cells; and (ii)
56 O₂-sensitive N₂ fixation in heterocysts. This ability has evolved through cellular differentiation
57 under nitrogen limiting growth conditions when some vegetative cells from the filament
58 transform into specialized heterocyst cells that provide a microaerobic environment suitable
59 for N₂ fixation. H₂ gas is naturally produced as an obligatory by-product of the N₂-fixation
60 process carried out by nitrogenase, which is highly sensitive to O₂. The natural yield of H₂ gas
61 production inside heterocysts is limited. This is due to rapid H₂ recycling, mainly by an uptake
62 hydrogenase enzyme, which further returns electrons for the N₂-fixing metabolism (Tsygankov
63 et al., 2007; Bothe et al., 2010).

64 In oxygenic photosynthesis, light drives the photosynthetic linear electron transport from water
65 to NADPH, using Photosystem (PS) II, Cytochrome (Cyt) *b₆f* and PSI complexes embedded in
66 the thylakoid membrane. These electron transport reactions are coupled to ATP synthesis *via*
67 the generation of trans-thylakoid proton motive force (*pmf*). The obtained NADPH and ATP
68 are then used as reducing power for CO₂ fixation and cell metabolism. Environmental
69 fluctuations in light and nutrient supply might result in the over-reduction of the photosynthetic
70 machinery. Alleviation of excess electrons by the class-C Flavodiiron proteins (hereafter FDP)
71 has been described in all oxygenic photosynthetic organisms, apart from angiosperms, red and
72 brown algae (Helman et al., 2003; Zhang et al., 2009; Jokel et al., 2018; Gerotto et al., 2016;
73 Chaux et al., 2017; Ilik et al., 2017; Alboresi et al., 2019; Shimakawa et al., 2021). This group
74 of proteins act as strong electron outlets downstream of PSI by catalyzing the photoreduction
75 of O₂ into H₂O (named the Mehler-like reaction) (Allahverdiyeva et al., 2011; 2013; 2015;
76 Santana-Sánchez et al., 2019).

77 Six genes encoding FDPs have been reported in *Anabaena* (Ow et al., 2008; Zhang et al., 2009;
78 Ermakova et al., 2013; Allahverdiyeva et al., 2015). Phylogenetic assessment has shown that
79 four of these genes (*flv1A*, *flv3A*, *flv2*, and *flv4*) are highly similar to their homologs in
80 *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), SynFlv1-SynFlv4. Recently, we
81 demonstrated that SynFlv1 and SynFlv3 proteins function in coordination with, but distinctly
82 from SynFlv2 and SynFlv4 (Santana-Sánchez et al., 2019). While the SynFlv1/Flv3 hetero-
83 oligomer is mainly responsible for the initial fast and transient O₂ photoreduction during a

sudden increase in light intensity, SynFlv2/Flv4 catalyzes steady O₂ photoreduction under illumination at air-level CO₂ (LC). Importantly, the single deletion of any FDP strongly diminishes the O₂-photoreduction, indicating that O₂ photoreduction is mainly catalyzed by the hetero-oligomeric forms working in an interdependent manner (Santana-Sánchez et al., 2019; Nikkanen et al., 2020).

The two additional *Anabaena* FDP proteins, AnaFlv1B and AnaFlv3B, are exclusively localized in the heterocysts (Ermakova et al., 2013). The AnaFlv3B protein was shown to mediate the photoreduction of O₂ independently of AnaFlv1B, likely as a homo-oligomer, playing an important role in maintaining micro-oxic conditions inside heterocysts under illumination, which is crucial for N₂ fixation and H₂ production (Ermakova et al., 2014). However, research on the role of heterocyst-specific AnaFlv1B and vegetative cell-specific FDPs in diazotrophic cyanobacteria is still scarce.

Here, we addressed the physiological relevance of the AnaFlv1A and AnaFlv3A isoforms on the bioenergetic processes occurring in vegetative cells and heterocysts of diazotrophic *Anabaena*. AnaFlv1A and AnaFlv3A were shown to have a crucial photoprotective role under fluctuating light intensities (FL), regardless of nitrogen or CO₂ availability, suggesting functional analogy with homologs in *Synechocystis*. Importantly however, our results also provided evidence for distinct functional roles of AnaFlv3A and AnaFlv1A. We showed that by cooperating with AnaFlv2 and/or AnaFlv4, AnaFlv3A can function independently of AnaFlv1A in O₂ photoreduction in low CO₂ conditions. AnaFlv3A was also indirectly linked with the H₂ metabolism occurring inside heterocyst cells. Our work highlights the complex regulatory network between oxygenic photosynthesis, nitrogen fixation and hydrogen photoproduction.

Materials and Methods

Strains and culture conditions

Anabaena sp. PCC 7120 strain was used as the wild-type (WT) in this study. The $\Delta flv1A$ and $\Delta flv3A$ mutants (Allahverdiyeva et al., 2013) and the $\Delta hupL$ mutant (Masukawa et al., 2002) were previously reported. For construction of the double mutant $\Delta flv1A/flv3A$, the BamHI-XbaI region of the mutated *flv1A* construct was replaced with the spectinomycin/streptomycin

resistance cassette. The generated plasmid was transferred into $\Delta flv3A$ and sucrose, neomycin, and spectinomycin was used for selection. Segregation of the mutant was verified by PCR. Culture stocks of $\Delta flv1A$ and $\Delta flv3A$ mutants were maintained in BG-11 medium supplemented with 40 $\mu\text{g mL}^{-1}$ neomycin, while the $\Delta hupL$ mutant was supplemented with 20 $\mu\text{g mL}^{-1}$ spectinomycin.

Pre-cultures were grown in Z8x medium (lacking combined nitrogen, pH 7.0-7.3, Kotai, 1972) at 30 °C and under constant white light of 75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ without antibiotics. For this, the filaments were inoculated at $\text{OD}_{750}=0.1$ in 200 mL Z8x medium (in 500 mL flasks) and were continuously bubbled with air (0.04% CO_2 , LC) or with air supplemented with 1% CO_2 (HC) if not specifically mentioned. Pre-cultures were harvested at the logarithmic growth phase, inoculated at $\text{OD}_{750}=0.1$ in fresh Z8x medium and experimental cultures were grown under similar pre-experimental conditions (75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination and bubbling with air or 1% CO_2 supplemented). Experimental cultures were harvested after 4 days of growth and experiments were conducted in 3-5 independent biological replicates.

Determination of heterocyst frequency

Alcian blue was used to stain the polysaccharide layer of the heterocyst envelope. Cell suspensions were mixed (1:8) with a solution of 0.5% Alcian Blue stain in 50% ethanol-water. Stained samples were visualized using a Wetzlar light microscope (Leitz) and x400 magnification micrographs were taken. Around 1000-2000 cells were counted per sample, and the heterocyst frequency was determined as a percentage of total cells counted.

MIMS measurements

In vivo measurements of $^{16}\text{O}_2$ ($m/z = 32$), $^{18}\text{O}_2$ ($m/z = 36$), CO_2 ($m/z = 44$) and H_2 ($m/z = 2$) fluxes were monitored using a membrane inlet mass spectrometry (MIMS) as described previously (Mustila et al., 2016). Harvested filaments were resuspended with fresh Z8x medium, adjusted to Chl *a* 10 $\mu\text{g mL}^{-1}$ and acclimated for 1 hr to the growth conditions. For LC samples, the concentration of dissolved total inorganic carbon was saturated with 1.5 mM NaHCO_3 before the measurement.

To measure Deuterium uptake, the filaments were flushed with Ar inside gas-tight vials for 15 min, then 1.2 mL pure D_2 (2 % in headspace) was injected into each vial. Changes of D_2 in the gas phase were measured at 2 h and 24 h after D_2 addition. 250 μL gas sample from the

headspace of the vials was injected into the MIMS chamber. The calibration of D₂ concentration was performed by injecting known concentrations of D₂ into the media.

Fluorescence analysis

A pulse amplitude modulated fluorometer Dual-PAM-100 (Walz) was used to monitor Chl *a* fluorescence and P700 absorbance. Harvested filaments were resuspended in fresh Z8x medium to the Chl *a* concentration of 15 µg mL⁻¹ and then kept for about 1 hr under the growth conditions. Before the measurements, samples were dark-adapted for 10 min. The measurement started with a saturating pulse in darkness to determine F_m^D. Then, the samples were illuminated with red actinic light at a photon flux density of 50 µmol photons m⁻² s⁻¹ for 380 s whilst saturating pulses (SP, 5000 µmol photons m⁻² s⁻¹, 400 ms) were given every minute (SP1-SP9). Photosynthetic parameters were determined as described previously (Huokko et al 2017).

Determination of PSI primary donor (P700) and Ferredoxin (Fd) redox changes from near-infrared absorbance

The absorbance differences at 780–820 nm, 820–870 nm, 840–965 nm and 870–965 nm were measured with the Dual KLAS/NIR spectrophotometer (Walz). Experimental cultures, as well as cultures used for determination of the model spectra (more info in Supplementary Methods), were grown at 50 µmol photons m⁻² s⁻¹ and under air-level CO₂ (LC) in Z8x medium for 4 days, then adjusted to Chl *a* concentration of 20 µg mL⁻¹ by re-inoculating pelleted cells in fresh medium. Cells were dark-adapted for 10 min, after which absorbance differences of the four wavelength pairs were measured during 5 s actinic illumination at 500 µmol photons m⁻² s⁻¹ and subsequent dark. The maximal levels of P700 oxidation and Fd reduction were determined for each sample by utilizing the NIRMAL script (Klughammer and Schreiber 2016), and the obtained experimental deconvoluted traces were then normalized to the maximal values. The Dual-KLAS/NIR measurement of *Synechocystis* Δ*flv1* cells was performed as described previously (Nikkanen et al., 2020).

H₂ measurement by Clark-type electrode

H₂ concentration was monitored under anaerobic conditions using a Clark-type Pt-Ag/AgCl electrode chamber (DW1/AD, Hansatech) connected to a homemade polarographic box.

Experimental cultures were harvested, resuspended in fresh Z8x medium and adjusted to the Chl *a* concentration of about 3-4 $\mu\text{g mL}^{-1}$. The resulting suspensions (~30 mL) were transferred into 75 mL glass vials, sealed and sparged with either nitrogen (N_2) or argon (Ar) for 30 min in the dark to achieve anaerobic conditions. Then, cultures were incubated under the corresponding atmosphere for another 2 h in the dark at 25 °C. 4 mL of dark-adapted suspension were transferred into the chamber with an anaerobic gas-tight syringe and H_2 concentration was monitored during 6 min illumination with actinic light of 800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ after which the light was switched off. The H_2 production rates were calculated using linear regression.

Nitrogenase activity essay

Acetylene reduction assay was used to determine nitrogenase activity as described previously (Leino et al. 2014). 5 mL of experimental samples were transferred into 23 mL vials, flushed with argon for 20 min and supplemented with 10% acetylene in the headspace. Vials were kept for 20 hr under 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 30 °C with gentle agitation (120 rpm). After this, 20 μL of gas sample was withdrawn from the headspace of the vial and analysed for ethylene content using a gas chromatograph equipped with Carboxen®-1010 PLOT Capillary Column and FID detector. The enzyme activity was calculated from the peak area and normalised to the total protein content.

Chl *a* and total sugar determination

Chl *a* was extracted from cells in 90% methanol and the concentration was determined by measuring absorbance at 665 nm and multiplying it with the extinction coefficient factor 12.7 (Meeks & Castenholz, 1971). For total sugar determination, 1 mL of experimental samples were collected, washed and diluted to 1:1 with MQ-water before the sugar measurement. Total sugar content was obtained using the colourimetric method described earlier (DuBois et al., 1956).

Protein extraction and immunoblotting

Total protein extracts were isolated as described previously (Zhang et al., 2009). Electrophoresis and immunoblotting were performed according to an earlier report (Mustila et al., 2016). Protein-specific antibodies raised against Flv3A (Agrisera), PsaB (AS10 695,

Agrisera), NdhK (Agrisera), and HupL (kindly provided by P. Tamagnini) were used in this study.

RNA isolation and RT-qPCR analysis

Isolation of total RNA, reverse transcription and qPCR analysis was performed as described earlier (Ermakova et al., 2013). *rnpB* gene was used as a reference for normalization. The primer pairs used in this study are listed in Table S1.

Results

Phenotypic characterization of *Anabaena* mutants deficient in Flv1A and Flv3A

To investigate the function of the vegetative cell-specific Flv1A and Flv3A proteins in diazotrophic *Anabaena* filaments, we used $\Delta flv1A$ and $\Delta flv3A$ deletion mutants (Fig. S1 and Allahverdiyeva et al., 2013). Likewise, the SynFlv1 and SynFlv3, the AnaFlv1A (encoded by *all3891*) and AnaFlv3A (encoded by *all3895*) proteins are indispensable for diazotrophic and non-diazotrophic growth of *Anabaena* filaments under severe fluctuating light intensities at both air level (low CO₂, LC) and 1-3 % CO₂ (high CO₂, HC) (Fig. S2a, Allahverdiyeva et al., 2013).

Under constant light (at a photon flux density of 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), there were no significant differences in the growth of these mutants compared to the WT, as measured by OD₇₅₀ (Fig. S2b) or concentration of chlorophyll *a* (Chl *a*). Total protein and sugar content of the WT and $\Delta flv1A$ and $\Delta flv3A$ filaments were also similar (Table 1). Light microscopic images of *Anabaena* filaments indicated that both $\Delta flv1A$ and $\Delta flv3A$ mutants and WT had a similar ratio of vegetative cells to heterocysts (Table 1) and no visible changes were observed in heterocyst morphology.

Table 1. Growth characteristics and photosynthetic parameters of the WT, $\Delta flv1A$, and $\Delta flv3A$ filaments. Experimental cultures were grown under diazotrophic LC conditions for 4 days. The maximum quantum yield of PSII (Fv/Fm), minimal level of fluorescence (Fo), maximal fluorescence in the dark (F_m^D), maximal fluorescence (F_m[']), quenching due to state transition (qT). Values are means $\pm$ SD, n = 3-5 biological replicates. Asterisks indicate statistically significant differences compared to the WT (t-test, *P* < 0.05).

Parameters	WT	$\Delta flv1A$	$\Delta flv3A$
OD ₇₅₀	1.46±0.18	1.45±0.13	1.43±0.18
Chl <i>a</i> , µg mL ⁻¹	7.01±0.80	6.66±0.76	6.97±0.96
Total protein, µg mL ⁻¹	263.6±31.7	265.3±10.1	254±14.1
Total sugars, µg mL ⁻¹	53.8±12.7	45.4±4.9	43.9±5.8
Heterocyst frequency (%)	12.7±1.1	12.1±0.9	10.7±2.1
Nitrogenase activity (C ₂ H ₂ reduction), µmol mg Chl _a ⁻¹ h ⁻¹	21.3±4.0	16.7±4.6	15.9±2.7*
F _v /F _m (with 10 µM DCMU)	0.45±0.05	0.5±0.01	0.49±0.01
F _o	0.91±0.01	0.86±0.00*	0.84±0.00*
F _m ^D	1.39±0.02	1.25±0.02*	1.26±0.01*
F _m ' (SP 1)	1.46±0.05	1.42±0.04	1.49±0.04
qT	0.05±0.02	0.14±0.01*	0.18±0.01*

230

231 Fluorescence and P700 oxidoreduction properties of $\Delta flv1A$ and $\Delta flv3A$

232 Diazotrophic *Anabaena* WT, $\Delta flv1A$, and $\Delta flv3A$ filaments, grown under LC and HC, were next
 233 subjected to fluorescence analyses under growth light intensity to disclose the impact of Flv1A
 234 and Flv3A, common or specific, on photosynthetic electron transport. The dark-adapted WT
 235 filaments showed a relatively low maximal fluorescence in the dark (F_m^D) (state 2). Upon
 236 exposure to actinic light intensity, maximal fluorescence (F_m') slightly increased, indicating a
 237 transition of filaments to state 1 (Fig. 1a). The state 2-to-state 1 transition observed upon
 238 illumination was less pronounced in HC-grown WT filaments (Fig. 1b). The effective yield of
 239 PSII [Y(II)], calculated for each saturating pulse (SP), remained stable (0.34±0.02 - 0.30±0.01)
 240 during the illumination of WT filaments grown both under LC (Fig. 1c) and HC (Fig. 1d)
 241 conditions.

242 Both the $\Delta flv1A$ and $\Delta flv3A$ mutants showed significantly lower F_m^D than WT in LC (Fig. 1
 243 and Table 1), implying a more pronounced state 2 in the dark. Accordingly, a stronger state 2-
 244 to-state 1 transition (qT in Table 1) was observed during illumination in comparison to WT,
 245 similarly to the phenotype previously described in the *Synechocystis* $\Delta flv3$ mutant (Elanskaya
 246 et al., 2021). Notably, during the dark-to-light transition, the fluorescence kinetics were

247 differently affected in the two mutants grown under LC conditions. Illumination of $\Delta flv3A$
 248 filaments resulted in a rapid increase of the fluorescence level which was gradually quenched
 249 but remained at a higher steady-state level (F_s) compared to WT and the $\Delta flv1A$ mutant. The
 250 $\Delta flv1A$ mutant showed only a moderate increase and then a gradual decay of fluorescence,
 251 reaching the WT F_s level after 4 min of illumination. Differently from LC grown filaments, the
 252 $\Delta flv1A$ and $\Delta flv3A$ mutants grown under HC conditions revealed a similar fluorescence
 253 increase during the dark-to-light transition, which gradually decayed and reached the WT levels
 254 by the end of the illumination period (Fig. 1b).

Figure 1. Fluorescence analysis of the diazotrophic *Anabaena* WT, $\Delta flv1A$, and $\Delta flv3A$. (A, C) Filaments were cultivated under air (LC) or (B, D) under air supplemented with 1% CO₂ (HC). Representative traces of 3 biological replicates are shown (A, B). Cells were dark acclimated for 10 min before illumination with 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of actinic light. The effective yield of PSII [Y(II)] was calculated by applying saturating pulses during induction curve measurements (C, D). Values are means $\pm$ SD, $n = 3$ biological replicates. Asterisks indicate statistically significant differences compared to the WT (t-test, $P < 0.05$). r.u., relative units.

255

256 The effective yield of PSII, Y(II) echoed high fluorescence levels and small variable
 257 fluorescence (F_v') upon illumination by SP1, showing a strong drop both in LC- (84% and 62%
 258 that of WT in $\Delta flv1A$ and $\Delta flv3A$ mutants, respectively) and HC-grown filaments (53% and
 259 39% that of WT in $\Delta flv1A$ and $\Delta flv3A$ mutants, respectively) (Fig. 1c,d). After that, the Y(II)
 260 values gradually recovered over the course of illumination, though $\Delta flv3A$ did not reach the

WT levels (Fig. 1c). Notably, the maximum quantum yield of PSII, F_v/F_m , did not differ significantly between the mutants and the WT (Table 1).

Examination of the transient post-illumination increase of fluorescence level (F_0 rise), which reflects the NDH-1 mediated reduction of the PQ pool in darkness (Mi et al., 1995), demonstrated a notably higher F_0 rise in both $\Delta flv1A$ and $\Delta flv3A$ mutants grown under LC and HC conditions (Fig. S3). In line with the lower F_m^D (Table 1), this finding suggests elevated electron flux into the PQ pool in the $\Delta flv1A$ and $\Delta flv3A$ mutants, presumably mediated by NDH-1, in comparison to WT. Considering that the abundance of NdhK, the core subunit of the NDH-1 complex, was similar between all genotypes (Fig. S4d) the difference in F_0 rise is likely caused by higher availability of reduced ferredoxin (Fd), the likely electron donor to both FDPs and NDH-1 (Nikkanen et al., 2021), or by post-translational regulatory factors.

At the onset of high irradiance both the $\Delta flv1$ (Fig. S5) and $\Delta flv3$ mutants of *Synechocystis* are unable to rapidly re-oxidize Fd, causing accumulation of electrons at P700 (Nikkanen et al., 2020; Theune et al., 2021). To determine whether this occurs in the *Anabaena* $\Delta flv1A$ and $\Delta flv3A$ mutants, we determined the high light-induced fast redox changes of Fd and P700 from near-infrared absorbance differences using the Dual KLAS/NIR spectrophotometer. The results indicated that similarly to *Synechocystis* $\Delta flv3$ (Nikkanen et al., 2020) and $\Delta flv1$ mutants (Fig. S5), both *Anabaena* mutants also suffered from delayed re-oxidation of Fd and P700 upon illumination, and showed slower post-illumination re-oxidation of Fd (Fig. 2). Unlike in the *Synechocystis* mutants, there was a clear difference between the $\Delta flv1A$ and $\Delta flv3A$ mutants of *Anabaena*, with the $\Delta flv3A$ strain displaying more severe delay in re-oxidation of Fd and P700 than $\Delta flv1A$.

Figure 2. Redox changes of P700 and Fd upon dark-light-dark transitions in the diazotrophic *Anabaena* WT, $\Delta flv1A$, and $\Delta flv3A$ filaments. The cells were grown under $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and air-level CO_2 (LC) for 4 days, harvested and adjusted to Chl *a* concentration of $20 \mu\text{g mL}^{-1}$. Cells were dark-adapted for 10 min, after which absorbance differences of four near-infrared wavelength pairs were measured with the Dual KLAS/NIR spectrophotometer during 5 s actinic illumination at $503 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and subsequent darkness. P700 and Fd redox changes were then deconvoluted from the absorbance differences using specifically determined differential model plots (model spectra) for *Anabaena* (see Materials and methods). Maximal levels of Fd reduction and P700 oxidation in each sample were used to normalize the traces. Representative traces of 3 biological replicates are shown.

284 **Real-time gas exchanges in *Anabaena* FDP mutants**

285 To clarify the specific impacts of *flv1A* and *flv3A* deletions on real-time gas fluxes in the
 286 diazotrophic filaments of *Anabaena*, we used membrane inlet mass spectrometry (MIMS)
 287 analysis (Fig. 3). The MIMS technique combined with the use of $^{18}\text{O}_2$ isotopologue allows
 288 distinguishing between light-induced O_2 reduction (uptake) and photosynthetic O_2 production.
 289 The net O_2 evolution rate was calculated as the difference between the rates of gross O_2
 290 evolution and O_2 uptake in the light. For all MIMS measurements, gas exchange was monitored
 291 for 4 min in dark followed by 5 min of high irradiance ($500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and for an
 292 additional 3 min in the dark.

293

Figure 3. O₂ and H₂ exchange rates of the diazotrophic *Anabaena* WT, $\Delta flv1A$ and $\Delta flv3A$ filaments. The filaments were cultivated for 4 days under air (LC) (A) and high carbon (1% CO₂ in the air, HC) (B), after which the filaments were harvested and the Chl a concentration adjusted to 10 $\mu\text{g mL}^{-1}$. Gas exchange rates were calculated in darkness (grey areas of the graphs) and under illumination with actinic white light at 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For LC measurements, samples were supplemented with 1.5 mM NaHCO₃. Orange shading indicates the differences in the initial slope of the O₂ photoreduction rates. The plots are representative of three independent biological replicates.

Illumination of WT filaments grown under LC demonstrated a rapid increase in the rate of O₂ uptake from $10.6 \pm 2.7 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ in darkness to $34.6 \pm 0.2 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ in light. This fast induction phase was followed by a decay that stabilized at $28.4 \pm 1.4 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ after 3 min (Fig. 3a, Table S2). This pattern resembles, to some extent, previously described triphasic kinetics of O₂ photoreduction in *Synechocystis* grown under LC conditions (Santana-Sánchez et al., 2019), and in HC-grown *Chlamydomonas reinhardtii* cells illuminated with high light intensity (Saroussi et al., 2019). Both mutants showed slightly lower O₂ uptake rates in darkness than the WT (Table S2) but the rate of O₂ consumption under illumination was affected to different extents in $\Delta flv1A$ and $\Delta flv3A$ filaments. The $\Delta flv3A$ mutant exhibited strong impairment of light-induced O₂ uptake, showing a maximal rate of $15.6 \pm 0.1 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ (52 % lower than the WT) at the onset of light, which declined to a residual rate of $4.3 \pm 0.2 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ by the end of illumination. In contrast to the *Synechocystis* $\Delta flv1$ mutant (Fig. S7) where O₂ reduction is almost fully eliminated, the *Anabaena* $\Delta flv1A$ filaments showed an intermediate phenotype whereby a maximum light-induced O₂ reduction rate of $22.2 \pm 2.8 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ (34% lower than WT) was observed, which declined to $15.5 \pm 1.2 \mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$ (Fig. 3a). Moreover, both $\Delta flv1A$ and $\Delta flv3A$ mutants showed slower activation of O₂ photoreduction, with a more pronounced lag-phase in $\Delta flv3A$ (Fig. 3a, orange shading). These results suggested that both AnaFlv1A and AnaFlv3A contribute to the Mehler-like reaction, but to a differing extent and presumably in different homo/hetero-oligomeric arrangements.

To clarify whether the homo-oligomers of AnaFlv1A in $\Delta flv3A$ and conversely, the homo-oligomers of AnaFlv3A in $\Delta flv1A$ mutants contribute to the observed O₂ photoreduction rates (Fig. 3a), we constructed a double mutant $\Delta flv1A/\Delta flv3A$ (Fig. S6). MIMS analysis revealed that concomitant inactivation of both *flv1A* and *flv3A* strongly inhibited the O₂ photoreduction in *Anabaena* filaments cultivated under LC conditions (Fig. 3a) suggesting either contribution of AnaFlv1 and AnaFlv3 homo-oligomers to O₂ photoreduction in the single mutants or involvement of AnaFlv2 and/or AnaFlv4 proteins in this process. Previous studies with *Synechocystis* cells (Zhang et al., 2009; Wang et al., 2004; Eisenhut et al., 2012; Santana-Sánchez et al., 2019) and non-diazotrophic *Anabaena* WT filaments (Ermakova et al., 2013) demonstrated high transcript abundance of *flv2* and *flv4* at LC. Therefore, we next investigated the abundance of *flv2* and *flv4* transcripts in diazotrophic *Anabaena* filaments grown under LC and HC using RT-qPCR. The $\Delta flv1A$ and $\Delta flv3A$ mutants grown under LC demonstrated significantly higher *flv2* and *flv4* transcript levels compared to the WT (Fig. S4a). Under HC,

transcript abundances of *flv2* and *flv4* did not differ between the mutants and the WT but were drastically lower in all genotypes compared to LC conditions (Fig. S4b). This prompted us to examine the possible contribution of AnaFlv2 and AnaFlv4 proteins to the Mehler-like reaction by comparing the O₂ photoreduction rates in $\Delta flv1A$ and $\Delta flv3A$ mutants grown under LC (Fig. 3a) vs HC conditions (Fig. 3b), where the expression of *flv2* and *flv4* were found to be induced and repressed, respectively.

While the O₂ photoreduction in WT filaments grown under HC was comparable to that under LC conditions (Fig. 3a), the inactivation of *flv1A* and/or *flv3A* fully eliminated light-induced O₂ reduction in the filaments grown under HC (Fig. 3b). This result suggests that the highly expressed *flv2* and *flv4* likely contribute to O₂ photoreduction in diazotrophic $\Delta flv1A$ and $\Delta flv3A$ filaments grown under LC conditions. Nevertheless, further elucidation is needed to verify the functioning of the AnaFlv2/Flv4 hetero-oligomer or different FDP oligomer compositions in O₂ photoreduction.

It is important to note that under LC conditions, while gross O₂ evolution and net photosynthetic O₂ production rates of the $\Delta flv1A$ mutant were comparable to those of the WT, the $\Delta flv3A$ mutant demonstrated lower gross and net O₂ evolution rates (Fig. 3a, Table S2). Strikingly, the initial peak in CO₂ uptake rates associated with the CCM activation (Liran et al., 2018) as well as the steady-state of CO₂ fixation of both deletion mutants were significantly diminished compared to the WT, and the $\Delta flv3A$ strain showed pronounced impairment than $\Delta flv1A$ (Table S2, Fig. S8b). Under HC conditions, both mutants had lower gross O₂ evolution (65.9 ± 7.3 and 76.8 ± 6.5 $\mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$, respectively) relative to the WT (122.2 ± 14.8 $\mu\text{mol O}_2 \text{ mg Chl } a^{-1} \text{ h}^{-1}$) and a delay in the induction of O₂ evolution upon illumination (Fig. 3b). Accordingly, the activation of CO₂ fixation under HC conditions was slower and decreased in both mutants compared to WT (Fig. S8c). In the $\Delta flv1A/\Delta flv3A$ double mutant a delay in gross O₂ evolution was observed under LC that was more severe than in $\Delta flv3A$ cells (Fig. 3A), while under HC all three mutant strains were similarly impaired (Fig. 3b). This suggests that not only AnaFlv3A, but also AnaFlv1A may be performing some AnaFlv2-4-dependent but AnaFlv3A-independent function in LC that affects photosynthetic electron transport.

Consequences of *flv1A* or *flv3A* deletion on diazotrophic metabolism

Based on the results above it is clear that AnaFlv1A and AnaFlv3A impact the photosynthetic apparatus to different extents in LC-grown diazotrophic *Anabaena*. In comparison to the

inactivation of AnaFlv1A, the deletion of AnaFlv3A resulted in a stronger reduction of the PQ pool, leading to a consistent decrease of PSII effective yield (Fig. 1c) and, eventually, lower net O₂ evolution rates over the illumination period (Fig. 3a). On the other hand, previous studies with different diazotrophic *Anabaena* species have demonstrated that the disruption of PSII activity in vegetative cells has implications for N₂ and H₂ metabolism inside heterocysts, thus, modulating the diazotrophic metabolism of filaments (Khetkorn et al., 2012; Chen et al., 2014). We, therefore, examined whether the absence of AnaFlv1A or AnaFlv3A from vegetative cells has a long-distance impact on the heterocyst metabolism. To this end, we analyzed the nitrogenase activity and H₂ fluxes of diazotrophic *Anabaena* WT, $\Delta flv1A$ and $\Delta flv3A$ mutants.

As demonstrated in Table 1, both $\Delta flv1A$ and $\Delta flv3A$ mutants showed somewhat lower nitrogenase activity in comparison to WT filaments, yet only the nitrogenase activity of the $\Delta flv3A$ mutant was significantly lower compared to WT. Real-time gas exchange monitored by MIMS (Fig. 3) revealed no changes in the H₂ gas concentration in WT and $\Delta flv1A$ during the dark-light transition. In contrast, the $\Delta flv3A$ mutant showed an increase in H₂ level in the dark and a clear light-induced H₂ gas production ($1.7 \pm 0.4 \mu\text{mol mg Chl } a^{-1} \text{ h}^{-1}$) (Fig. 3A). This result was confirmed by a second independent $\Delta flv3A$ mutant strain showing similar light-induced H₂ production ($\Delta flv3A_C2$ in Fig. S8a). Interestingly, the $\Delta flv3A$ mutant cultivated under HC conditions demonstrated an even higher H₂ photoproduction rate ($2.8 \pm 0.8 \mu\text{mol mg Chl } a^{-1} \text{ h}^{-1}$, Fig. 3b). Although $\Delta flv3A$ filaments showed real-time H₂ production under oxic conditions, the rate of H₂ production remained low.

Next, we monitored H₂ in anoxic cultures using a Clark-type electrode. Under the N₂ atmosphere, the $\Delta flv3A$ mutant demonstrated a significantly higher yield of H₂ photoproduction accompanied by a three times higher specific H₂ production rate compared to the WT (Fig. 4a, Fig. S9a). To confirm that the observed H₂ production is nitrogenase-mediated, we monitored the reaction under an argon (Ar) atmosphere as it is known that in the absence of N₂ substrate, nitrogenase reduces protons to H₂ (Hoffman et al., 2014). Indeed, the specific H₂ photoproduction rate of the WT filaments under an Ar was about 7-fold higher compared to the N₂ atmosphere (Fig. S9a). In the case of $\Delta flv3A$, the yield of H₂ photoproduction was strongly enhanced under an Ar and the production rate increased by around 10 times compared to N₂ (Fig. S9a). In addition, a drastically decreased transcript abundance of *hoxH* in both $\Delta flv1A$ and $\Delta flv3A$ mutants compared to the WT (Fig. S9b), implied a negligible contribution

of Hox to H₂ production in $\Delta flv3A$ mutant. Collectively, these results provide evidence that the enhanced H₂ production in the $\Delta flv3A$ mutant is mediated by nitrogenase.

However, the observed decrease in nitrogenase activity (Table 1) did not correlate with an increase in H₂ photoproduction in $\Delta flv3A$ (Fig. 3a). It is well known that the net nitrogenase-mediated production of H₂ in heterocysts is strongly affected by the activity of uptake hydrogenase (Hup), which oxidizes H₂ (Tamagnini et al., 2007). Therefore, it is conceivable that the impairment of the Hup function would account for the increased production of H₂ in $\Delta flv3A$. To examine the H₂ fluxes, we traced the uptake of Deuterium (²H₂, D₂) by the WT, $\Delta flv1A$, and $\Delta flv3A$. Whilst WT and $\Delta flv1A$ filaments efficiently consumed D₂, $\Delta flv3A$ showed a significantly lower capacity for D₂ uptake (Fig. 4B). These observations confirmed that the impaired capacity of the $\Delta flv3A$ mutant to recycle H₂ could be the reason for the increased accumulation of H₂ observed in $\Delta flv3A$ mutant.

To better understand the molecular mechanism behind the defective H₂ uptake of $\Delta flv3A$, we analyzed the transcript and protein abundances of the large subunit of Hup (HupL). We found significant downregulation of the mature form of *hupL* transcript in both $\Delta flv1A$ and $\Delta flv3A$ mutants, in comparison to the WT (Fig. 4c). Importantly, *hupL* transcript level in $\Delta flv3A$ was significantly lower than in the $\Delta flv1A$ mutant. Immunoblotting with specific antibody further revealed the lack of detectable HupL protein in $\Delta flv3A$, a result comparable to the *hupL*-disrupted mutant ($\Delta hupL$), while the $\Delta flv1A$ mutant showed only a lowered level of HupL relative to the WT (Fig. 4d). Taken together, these results demonstrate that the lack of HupL protein in heterocyst cells of $\Delta flv3A$ is the reason behind the enhanced nitrogenase based H₂ photoproduction observed for this mutant.

Figure 4. H₂ metabolism of diazotrophic filaments of *Anabaena* WT, $\Delta flv1A$, and $\Delta flv3A$. (A) H₂ production yield was monitored by a H₂ electrode under an Ar or N₂ atmosphere in the dark (grey areas) and under 800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light. (B) Deuterium uptake by the filaments was calculated from the difference in D₂ concentration between 2 h and 24 h after the injection in the vials initially flushed with Ar. (C) Relative transcript level of the mature *hupL*. (D) Immunodetection of HupL with a specific antibody. (E) Nitrogenase activity was measured using the acetylene reduction assay. Values are Mean $\pm$ SD, n = 3 biological replicates. Black asterisk indicates statistically significant differences compared to the WT (t-test, $P < 0.05$). Red asterisk indicates statistically significant differences compared to the $\Delta flv1A$ mutant (t-test, $P < 0.05$).

Discussion

The heterocyst-forming cyanobacteria are considered one of the earliest forms of multicellular filaments in the history of life. Despite the extensive characterization of heterocyst differentiation, little is known about the co-regulation and interdependence of the two contrasting processes of N₂ fixation and oxygenic photosynthesis occurring in heterocysts and vegetative cells, respectively. Under challenging environmental conditions, diazotrophic cyanobacteria must find an optimal balance between photochemical reactions and downstream processes that consume electrons in both cell types. In this work, we employed $\Delta flv1A$ and $\Delta flv3A$ mutants of *Anabaena* to examine the physiological significance of the vegetative cell-

specific AnaFlv1A and AnaFlv3A proteins on the bioenergetic processes of diazotrophic cyanobacteria. Our results provide evidence that, in contrast to the *Synechocystis* homolog, AnaFlv3A can mediate moderate O₂ photoreduction independently of AnaFlv1A and in coordination with AnaFlv2 and AnaFlv4 under LC conditions. Moreover, the vegetative-cell specific AnaFlv3A protein exhibits important link to the H₂ metabolism inside the heterocyst, since the inactivation of this protein results in high H₂ photoproduction even under ambient air. Nevertheless, we have demonstrated that both AnaFlv1A and AnaFlv3A proteins, presumably as hetero-oligomers, are required for efficient induction of the Mehler-like reaction during dark-to-light transitions, are crucial for photoprotection when light intensity rapidly fluctuates, and are likely needed for the activation of CO₂ assimilation.

In the absence of AnaFlv1A, AnaFlv3A can team up with AnaFlv2 and/or AnaFlv4 to mediate O₂ photoreduction under LC conditions

In line with previous transcriptional analysis showing a decrease in the expression of both *flv1A* and *flv3A* in *Anabaena* WT upon the shift to diazotrophic conditions (Ermakova et al. 2014), the single deletions of AnaFlv1A or AnaFlv3A did not affect the diazotrophic growth of mutants under continuous illumination compared to the WT (Table 1). However, both AnaFlv1A and AnaFlv3A proteins are indispensable during sudden changes in light intensity, similar to their homologous proteins in other species (Fig. S1a, Allahverdiyeva et al., 2013; Gerotto et al., 2016; Jokel et al., 2018). Here, we have demonstrated that when both AnaFlv1A and AnaFlv3A proteins are expressed in WT filaments, the rate of the Mehler-like reaction is rapidly increased during the dark-to-light transition likely due to the activity of the AnaFlv1A/Flv3A hetero-oligomer (Fig. 3). Accordingly, the absence of either AnaFlv1A or AnaFlv3A delays rise in O₂ photoreduction (Fig. 3a) resulting in over-reduction of the PQ pool upon illumination (Fig. 1a), causing a decrease in PSII yield (Fig. 1C) and impairment of PSI and Fd oxidation (Fig. 2). This phenotype is aggravated in the mutant lacking AnaFlv3A, which showed a stronger state 2-to-state 1 transition and more severe inability to oxidize PSI than the mutant lacking AnaFlv1A (Fig. 1 and Fig. 2). Differently from the *Synechocystis* $\Delta flv1$ mutant (Fig. S7), AnaFlv3A can promote O₂ photoreduction in the *Anabaena* $\Delta flv1A$ mutant (Fig. 3a), resulting in only about 45% inhibition of steady-state O₂ photoreduction and 35% decrease in Y(II) in *Anabaena* under LC growth conditions (Table S1). The near elimination of the steady-state O₂ photoreduction in the $\Delta flv1A/flv3A$ double mutant under LC and the single mutants under HC conditions (where AnaFlv2 and AnaFlv4 are strongly downregulated) supports (i)

functional AnaFlv3A/Flv2-4 oligomerization, and/or (ii) cooperation between the AnaFlv3A/Flv3A homo-oligomer and AnaFlv2/Flv4 hetero-oligomers. Accordingly, the strong impairment of O₂ photoreduction in $\Delta flv3A$ might be due to the inability of AnaFlv1A to function as a homo-oligomer and/or cooperate with AnaFlv2/Flv4. It is worth emphasizing that both $\Delta flv1A$ and $\Delta flv3A$ mutants showed similarly enhanced accumulation of *flv2* and *flv4* transcripts (Fig. S4a). While the $\Delta flv1A$ mutant displayed WT-like *flv3A* transcript and protein levels, the $\Delta flv3A$ mutant showed an elevated *flv1A* transcript level compared to the WT (Fig. S4c). This shows that the inhibition of O₂ photoreduction in $\Delta flv3A$ is not due to the downregulation of other FDPs. No contribution of the SynFlv3/Flv3 homo-oligomer in the Mehler-like reaction was observed *in vivo* (Mustila et al., 2016), contrary to previous *in vitro* studies suggesting a function of SynFlv3/Flv3 homo-oligomers in NAD(P)H-dependent O₂ reduction (Vicente et al., 2002, Brown et al., 2019). Instead, a possible photoprotective function of SynFlv3/Flv3 homo-oligomers *via* an unknown electron transport network was proposed (Mustila et al., 2016). In *Anabaena* $\Delta flv1A$ mutant, AnaFlv3A/Flv3A homo-oligomers may, for example, be involved in controlling the cation homeostasis, which in turn may affect the reversible association of AnaFlv2/Flv4 hetero-oligomers with the thylakoid membrane, and consequently, their involvement in O₂ photoreduction. It is also important to note that the oligomer formation scenario in the mutants might be different in *Anabaena* WT filaments. Overall, the obtained results suggest an important role for AnaFlv3A, but not AnaFlv1A, in mediating steady-state O₂ photoreduction under diazotrophic LC conditions in an AnaFlv2/Flv4-dependent. Moreover, in LC but not in HC conditions, the lack of both AnaFlv1A and AnaFlv3A resulted in a more severe delay in induction of O₂ evolution during dark-to-light transition in comparison to the lack of AnaFlv3A only (Fig. 3). This suggests that AnaFlv1A may also function in coordination with AnaFlv2/4 independently of Flv3A in an unknown role that facilitates photosynthetic electron transport. Understanding the exact functions of AnaFlv2 and/or AnaFlv4 in these processes and their interactions with AnaFlv1A and AnaFlv3A requires further investigation.

Even though AnaFlv1A and AnaFlv3A contribute to the Mehler-like reaction to different extents, both $\Delta flv1A$ and $\Delta flv3A$ mutants exhibited reduced CCM activity, as deduced from lowered initial peaks in CO₂ uptake rate during dark-to-light transition and reduction of steady-state CO₂ uptake in comparison to WT (Fig. S8b, Table S2). This is likely to result from impaired energization of CCM in the absence of AnaFlv1A or AnaFlv3A. SynFlv1 and SynFlv3 have been shown to have a crucial role in the generation of *pmf* during the dark-to-

light transition (Nikkanen et al., 2020), comparable to that of FLVA/B in *P. patens* (Gerotto et al., 2016) and *C. reinhardtii* (Chaux et al., 2017). Moreover, the *pmf* generated by FDPs and CET has been recently shown to be important for inducing and maintaining CCM activity in *C. reinhardtii* (Burlacot et al., 2021). We hypothesize that AnaFlv1A/Flv3A hetero-oligomer is required to rapidly induce the Mehler-like reaction, likely being important for the generation of *pmf* and possibly for induction of CCM activity during the dark-to-light transition. The molecular mechanism of the FDP-dependency of the CCM requires further investigation however, as the mechanisms of CCM differ between *Chlamydomonas* and cyanobacteria (Price et al 2008). Moreover, in *Synechocystis* mutants lacking Flv1/3 *pmf* generation during the first minute of dark-to-light transitions is severely impaired, CCM induction is largely unaffected at least in standard conditions (Nikkanen et al., 2020). In *Anabaena* however, both $\Delta flv1A$ and $\Delta flv3A$ strains demonstrated impaired induction of CCM (Fig. S8b), suggesting that *Anabaena* may differ from *Synechocystis* in the extent to which CCM induction is *pmf*-dependent.

Compelling evidence has recently been provided for dynamic coordination and functional redundancy between NDH-1 and SynFlv1/Flv3, jointly contributing to efficient oxidation of PSI in *Synechocystis* (Nikkanen et al., 2020) and in *Physcomitrella patens* (Storti et al., 2020a, 2020b). NDH-1-mediated cyclic electron transport (CET) in *Anabaena* could also partially compensate for a lack of AnaFlv1A and AnaFlv3A as evidenced by a stronger F_0 rise observed in both mutants (Fig. S3a). Unlike *Synechocystis* cells, *Anabaena* filaments express orthologs of plastid terminal oxidase (PTOX, *all2096*) (McDonald et al., 2003). It has been proposed that in *C. reinhardtii* and vascular plants, PTOX functions as an electron valve directing electrons from plastoquinol to O_2 , thereby controlling the redox state of the PQ pool (Stepien and Johnson, 2018, Saroussi et al., 2019; Nawrocki et al., 2019) and being involved in diverse metabolic processes such as the regulation of CET, state transition and carotenoid biosynthesis (Nawrocki et al., 2019). We cannot exclude possible contribution of PTOX to the residual O_2 photoreduction observed in the $\Delta flv3A$ mutant (Fig. 3A), and/or as a sensor of the redox state of the PQ pool and regulator of NDH-dependent CET, thus limiting electron pressure on the acceptor-side of PSI (Bolte et al., 2020).

Inactivation of AnaFlv3A leads to enhanced nitrogenase-based H_2 photoproduction under oxic conditions

Demonstration of elevated photoproduction of H₂ gas in diazotrophic filaments lacking vegetative cell-specific AnaFlv3A under oxic and microoxic conditions (Fig. 4) provided intriguing information about bioenergetic interdependence between vegetative cells and heterocysts. The heterocyst-originated production of H₂ in the $\Delta flv3A$ mutant was rapidly induced upon exposing the filaments to light and occurred concomitantly with the evolution of O₂ in neighbouring vegetative cells (Fig. 3). Moreover, the rate of H₂ photoproduction in the $\Delta flv3A$ mutant responded positively to an increase in CO₂ availability (Fig. 3b).

In the absence of N₂, the main substrate for nitrogenase, all electrons can be directed to H₂ production (Hoffman et al., 2014) allowing a less costly reaction, whereby only 4 moles of ATP are required to produce one mole of H₂. In this work, the removal of N₂ substrate (by replacement with Ar) led to a 10-fold increase of H₂ photoproduction rate in $\Delta flv3A$, demonstrating the occurrence of nitrogenase-dependent H₂ photoproduction in this mutant (Fig. 4a). A recent report suggested that overexpressing Flv3B lead to more stable microoxic conditions inside the heterocysts, notably increasing the H₂ production yield, presumably *via* the bidirectional hydrogenase Hox (Roumezi et al., 2020). In contrast to the unidirectional production of H₂ by nitrogenase, Hox catalyzes the reversible reduction of protons to H₂ (Bothe et al., 2010). We do not consider the contribution of Hox to the photoproduction of H₂ by the $\Delta flv3A$ mutant, as the net production does not fit with the bidirectional nature of the enzyme. Moreover, significant downregulation in the $\Delta flv3A$ mutant of transcripts from *hoxH*, encoding one of the subunits (Fig. S9b) further supports this assumption. Altogether, these results indicate that the increased light-induced H₂ photoreduction of the $\Delta flv3A$ mutant is mediated by nitrogenase activity.

Strikingly, it turned out that the increase in H₂ photoproduction yield in the $\Delta flv3A$ mutant was due to significant downregulation of HupL, the large subunit of the uptake hydrogenase, evidenced both at the transcript and protein levels (Fig. 4c, 4d). The absence of functional Hup suppressed the H₂ recycling pathway (Fig. 4b) and caused the release of H₂, photoproduced by nitrogenase, from the heterocysts of $\Delta flv3A$ filaments (Fig. 3, Fig. 4a). Thereby, our results highlight a regulatory network between the two metabolic processes in different compartments: The Flv3A-mediated metabolic processes in the vegetative cells and the H₂ metabolism in the heterocysts. It is likely that the redox state of the PQ pool in vegetative cells, affected by the activity of Flv3A, has a regulatory role on the H₂ metabolism in heterocysts. However, the nature of the molecular signal from reduced PQ that ultimately regulates gene expression in

heterocysts remains unknown. The redox state of the PQ pool is likely to correlate with the availability of soluble reducing cofactors in the cytosol of vegetative cells, and while evidence in *Anabaena* is lacking, those cofactors may be interchanged between vegetative cells and heterocysts, inducing changes in metabolism and gene expression. A majority of the NADPH needed for the nitrogen metabolism in heterocysts is understood to be derived from the oxidative pentose phosphate pathway breaking down carbohydrates imported from vegetative cells, (Cumino et al., 2007) but it is plausible that more direct exchange of cofactors also occurs, analogously to the malate redox shuttle between cytosol and the chloroplast in plants and algae. Nevertheless, the molecular mechanism underlying this regulatory network between different cell types needs further elucidation.

Taken together, our results demonstrate that similarly to SynFlv1 and SynFlv3, both vegetative-cells specific AnaFlv1A and AnaFlv3A are indispensable under harsh FL conditions regardless of nitrogen or CO₂ availability, most likely maintaining sufficient oxidation of the photosynthetic electron transport chain by catalysing the Mehler-like reaction as AnaFlv1A/Flv3A hetero-oligomers. Under LC, AnaFlv3A is able to perform moderate O₂ photoreduction in coordination with AnaFlv2 and AnaFlv4 proteins and independently of AnaFlv1A. AnaFlv3A may either stimulate the activity of AnaFlv2/Flv4 hetero-oligomers indirectly via an unknown function, or participate in forming functional hetero-oligomers with AnaFlv2 and Flv4. The deletion of AnaFlv3A was concomitant with the downregulation of the heterocyst-specific Hup enzyme resulting in increased bioproduction of H₂. This novel regulatory network between the photosynthesis and diazotrophic metabolism might represent an unexploited source for the future of biotechnological applications.

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Data availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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