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RESEARCH ARTICLE

Figures and figure supplements

Flavodiiron proteins 1–to-4 function in versatile combinations in O_2 photoreduction in cyanobacteria

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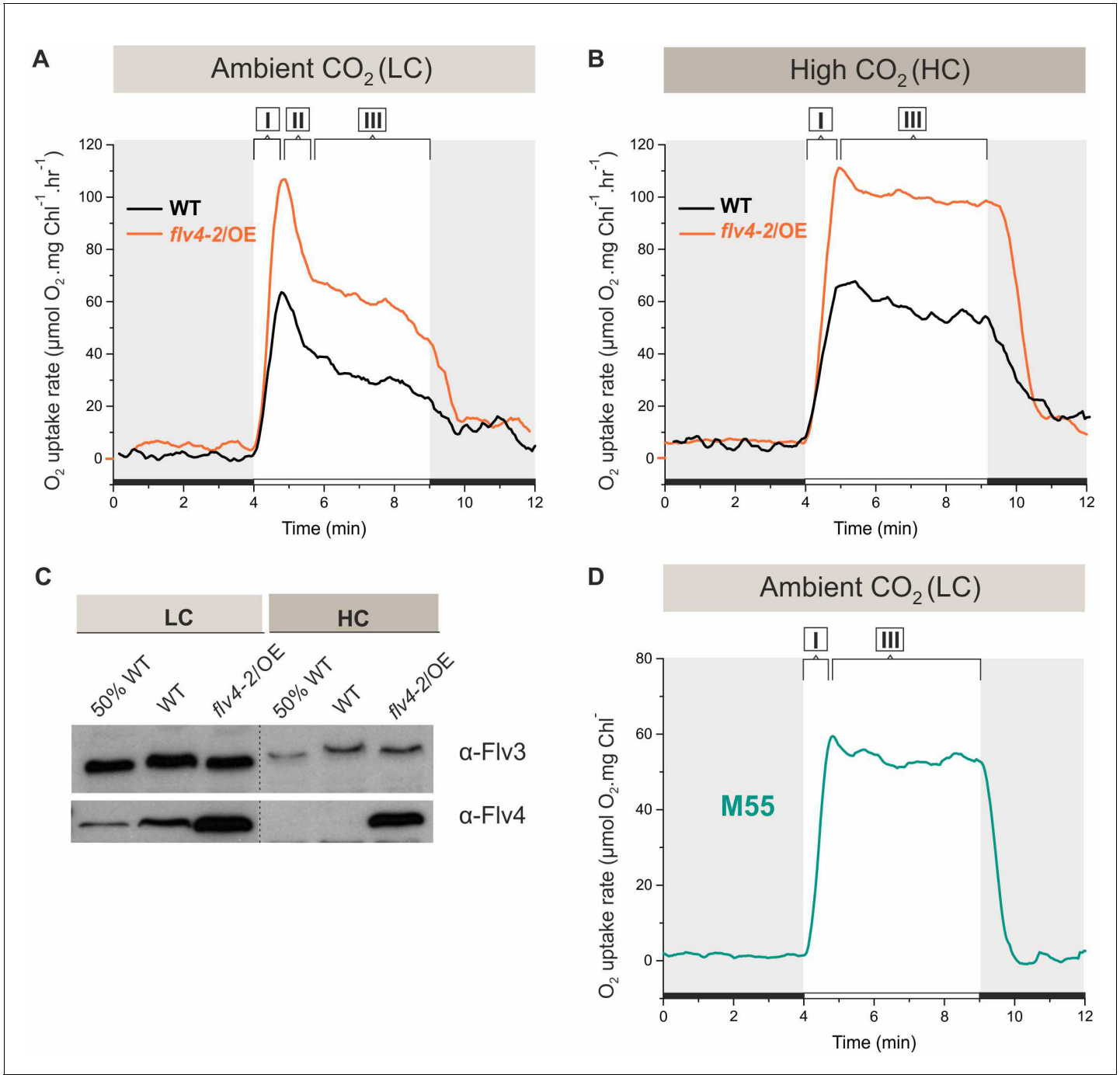

Figure 1. O_2 reduction rates and Flv3 and Flv4 protein accumulation in cells grown in low (LC) and high CO_2 (HC). (**A, B**) O_2 reduction rate of WT, *flv4-2/*OE and (**D**) the M55 mutant (Δ*ndhB)* was recorded in darkness (gray background) and under illumination (white background). The experiment was conducted in three independent biological replicates and a representative plot is shown. (***Figure 1—source data 1***). (**C**) Immunoblot detection of Flv3 and Flv4 in WT and *flv4-2/*OE. Pre-cultures were grown in BG-11, pH 8.2 under 3% CO_2 (HC) for 3 days, after that cells were harvested and resuspended in fresh BG-11, pH 8.2 at OD_{750} = 0.2. The experimental cultures were grown under HC or under LC. For the MIMS experiments the cells were harvested and resuspended in fresh BG-11, pH 8.2 at 10 µg Chl *a* mL^{-1}. O_2 photoreduction was recorded during the transition from darkness to high-light intensity of 500 µmol photons m^{-2}s^{-1}. In order to create comparable conditions for MIMS measurements, LC-grown cells were supplemented with 1.5 mM $NaHCO_3$ prior to the measurements. Independent experiments performed on WT cells grown in BG-11 lacking Na_2CO_3, but supplied with 1.5 mM $NaHCO_3$ prior to MIMS measurement showed no significant difference in O_2 photoreduction rates (***Figure 1—figure supplement 2***), thus allowing confident comparison of the MIMS results. Different phases of O_2 photoreduction kinetics are indicated as {I}, {II}, {III}. 50% WT, corresponds to 1:2 diluted WT total protein sample.

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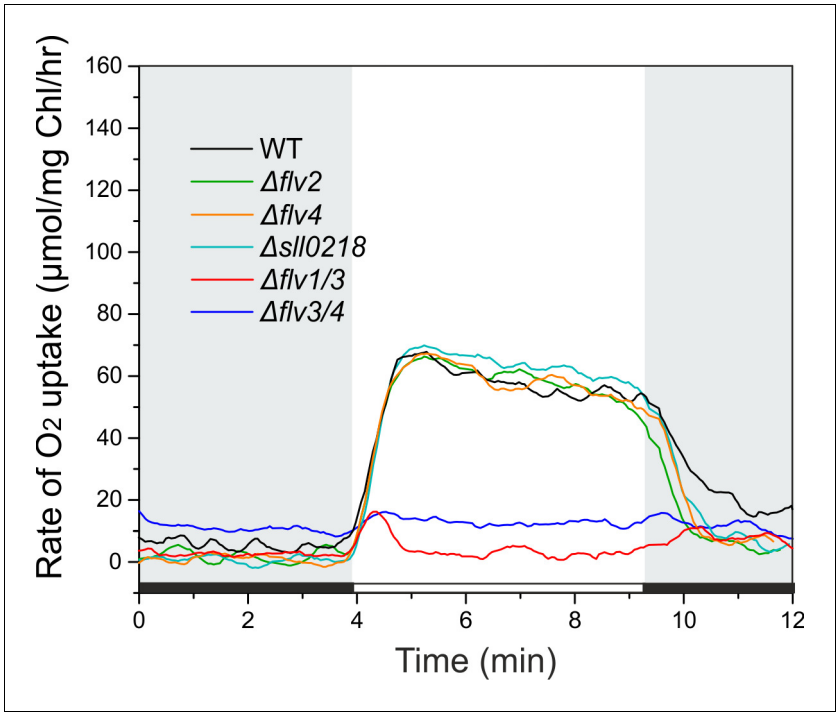

Figure 1—figure supplement 1. O_2 reduction rates under high CO_2. Cells were grown under 3% CO_2 (BG-11, pH 8.2), harvested and resuspended in fresh BG-11 at 10 µg Chl *a* mL^{-1} . O_2 uptake was recorded during the transition from dark to high-light (500 µmol photons m^{-2} s^{-1}).

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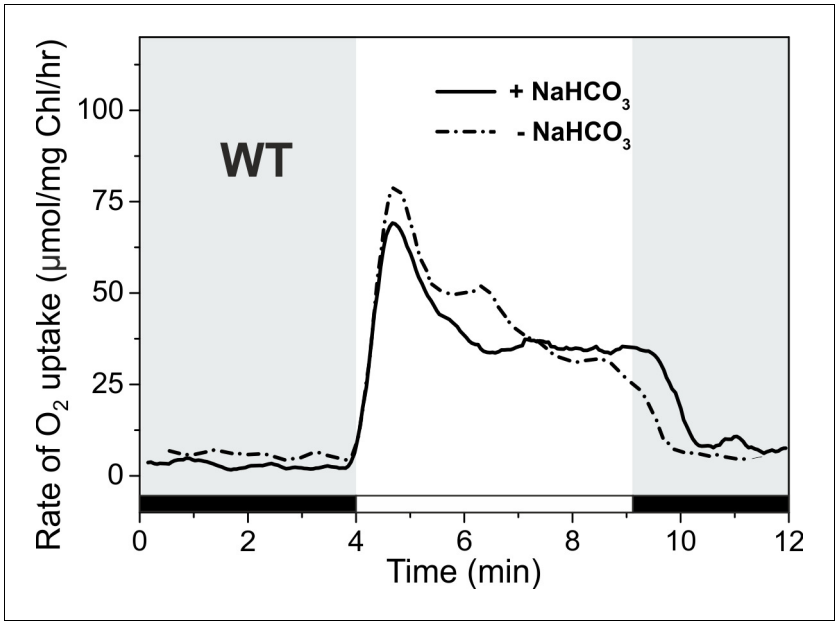

Figure 1—figure supplement 2. O_2 reduction rates during the dark-to-light transition of WT cells with and without addition of 1.5 mM $NaHCO_3$ prior MIMS measurements. The cells were harvested and inoculated in the fresh BG-11 7.5 without Na_2CO_3. Prior to MIMS measurement, cells were supplemented with 1.5 mM $NaHCO_3$ (solid line), or measured in the absence of an additional carbon source.

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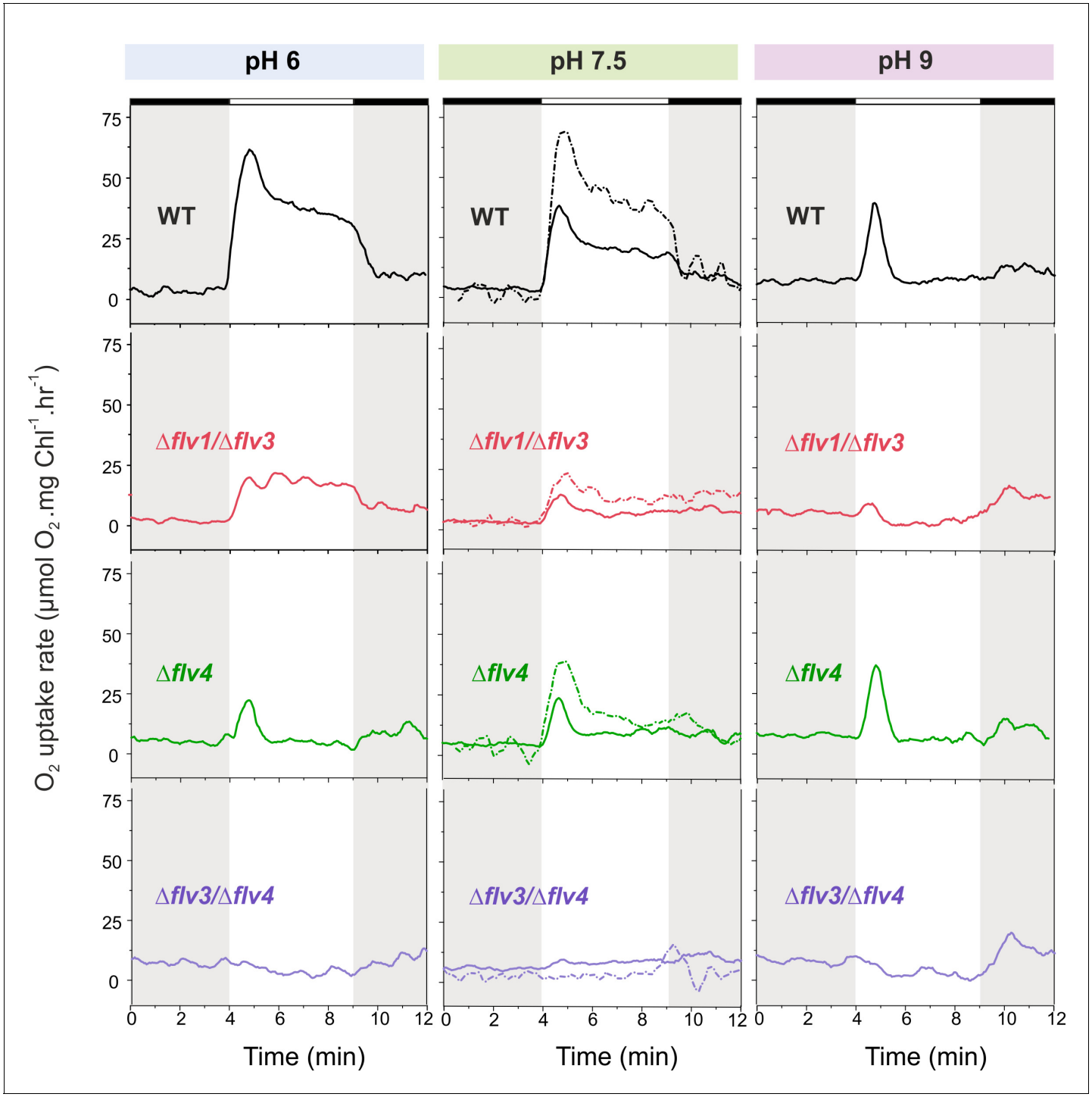

Figure 2. O_2 reduction rates of WT and FDP mutants grown at different pH levels. O_2 reduction rate was recorded in darkness (gray background) and under illumination with actinic white light at an intensity of 500 µmol photons m^{-2} s^{-1} (white background). Pre-cultures were grown in standard BG-11 medium (containing Na_2CO_3 at a final concentration of 0.189 mM) under HC for 3 days at different pH levels. For MIMS experiments, cells were shifted to LC at $OD_{750} \approx 0.2$ (same pH) and grown for 4 days before measurements. Exceptions were: (i) pH 6 experimental cultures were inoculated from pH 8.2 pre-cultures; and (ii) pH 7.5 pre-culture was shifted to LC in standard BG-11 containing Na_2CO_3 at a final concentration of 0.189 mM or in BG-11 without Na_2CO_3 (dotted line '- Na_2CO_3'). The experiment was conducted in three independent biological replicates (except experiment at pH 6 with n = 2 independent biological replicates) and a representative plot is shown. (***Figure 2—source data 1***). In order to create comparable conditions for MIMS measurements, all cells were supplemented with 1.5 mM $NaHCO_3$ prior to the measurements.

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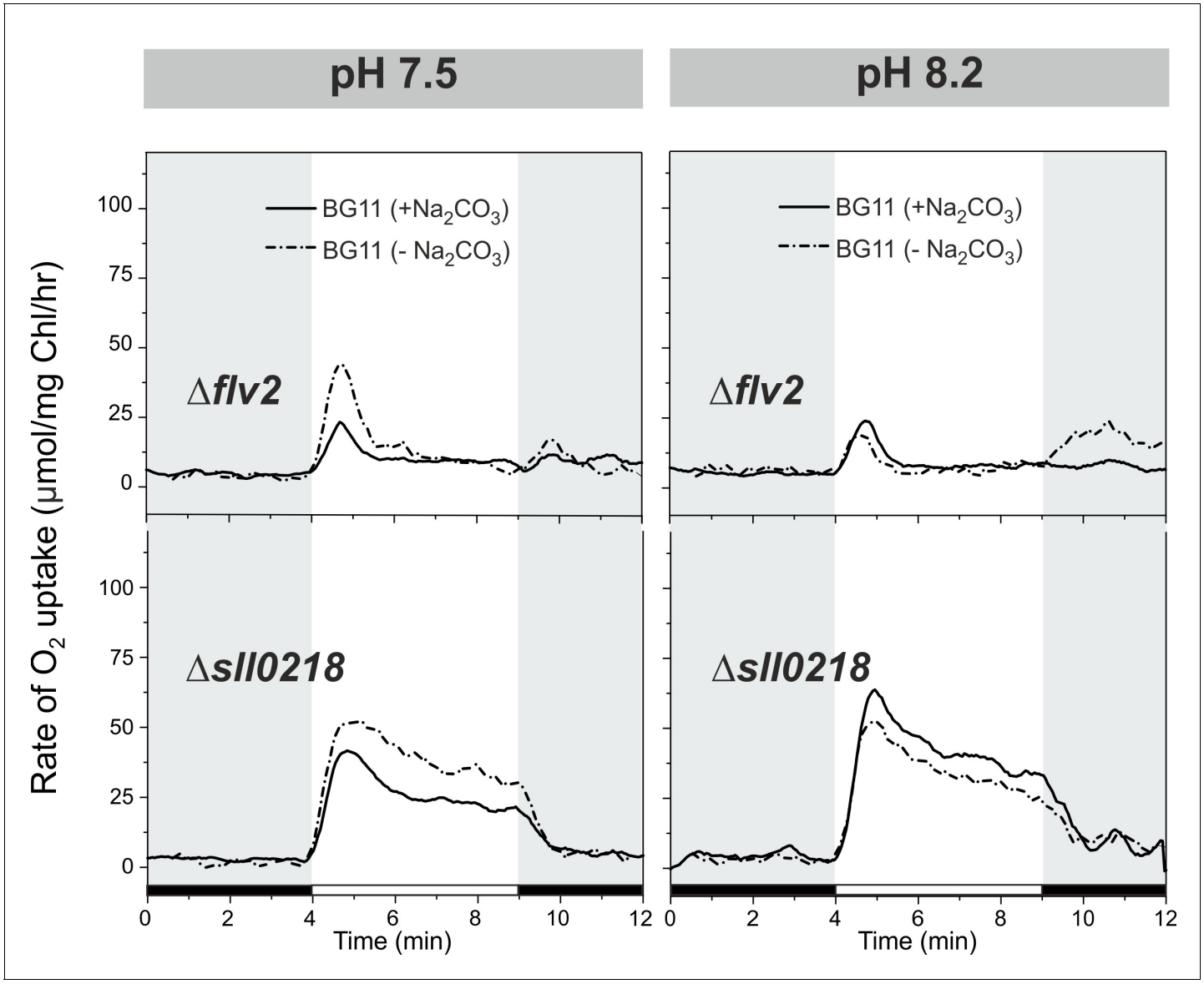

Figure 2—figure supplement 1. O_2 photoreduction rates of the Δ*flv2* and Δ*sll0218* mutants grown at LC pH 7.5 and 8.2 with and without Na_2CO_3. Pre-cultures were grown under HC for 3 days at pH 7.5 or pH 8.2 in BG-11 media with or without Na_2CO_3. For O_2 photoreduction experiments, cells were shifted to LC at $OD_{750} \approx 0.2$ and grown for 4 days.

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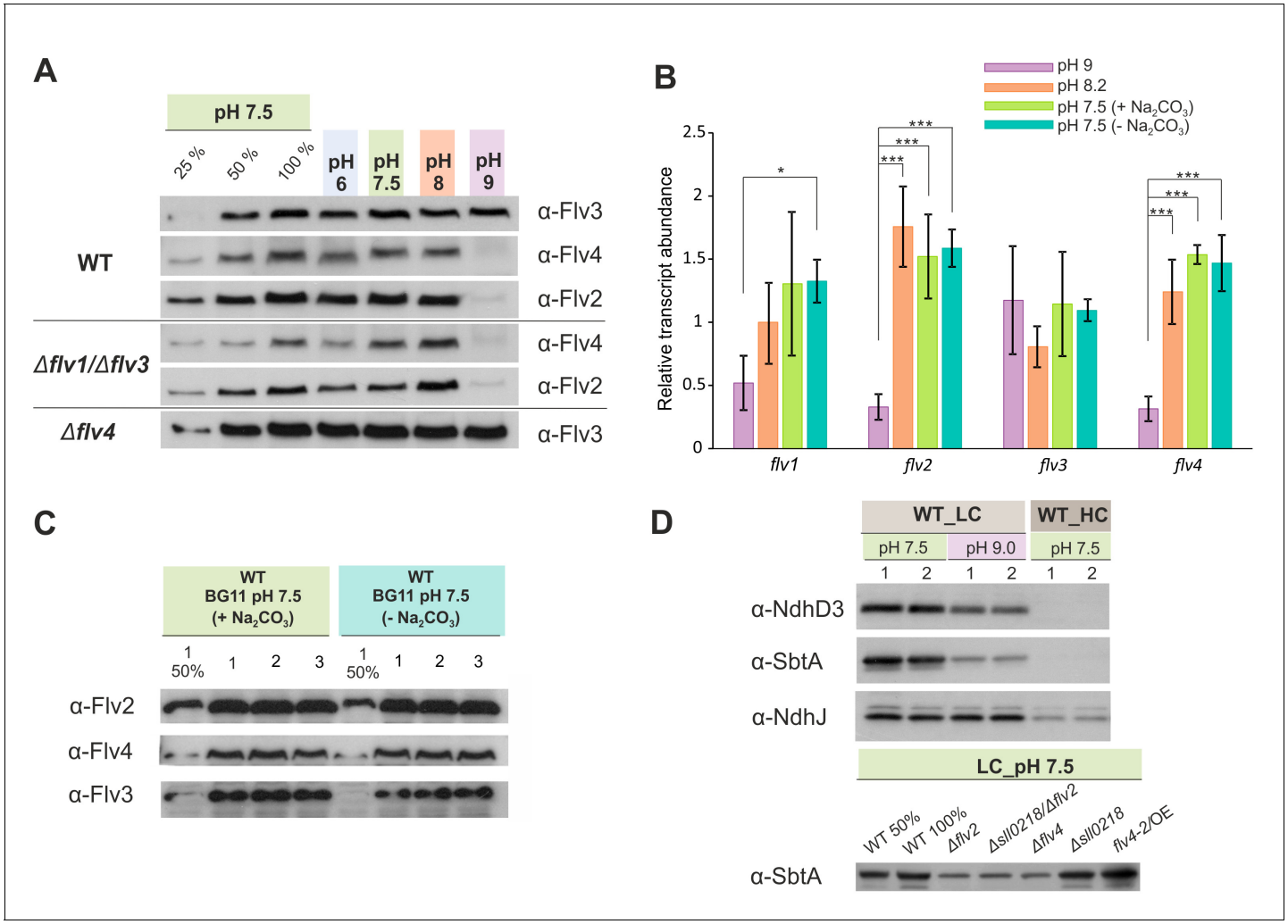

Figure 3. The effect of the pH of growth medium on the protein and transcript accumulation. (**A, B**) The effect of the pH and (**B, C**) sodium carbonate in the growth medium (**A, C**) on the protein and (**B**) transcript levels of FDP. (**D**) Protein immunoblots demonstrating the accumulation of bicarbonate transporter (SbtA) and NDH-1 subunits (NdhD3 and NdhJ) in the cells grown at different pH and CO_2 concentration. Cells were pre-grown at different pH levels (+Na_2CO_3) under HC for 3 days, harvested, resuspended in fresh BG-11 (pH maintained), adjusted to $OD_{750} \approx 0.2$ and shifted to LC for 4 days. At pH 7.5, the cells were grown at LC in the presence (+ Na_2CO_3, at final concentration of 0.189 mM) or in the absence (- Na_2CO_3) of sodium carbonate (**B, C**). Transcript abundance is presented as mean ± SD, n = 2–4 biological replicates, asterisks indicate a statistically significant difference to the WT (*p<0.05; ***p<0.001) (***Figure 3—source data 1***). Numbers 1–3 indicate different biological replicates. 25% and 50% correspond to 1:4, 1:2 diluted total protein sample, and 100% indicates undiluted total protein sample.

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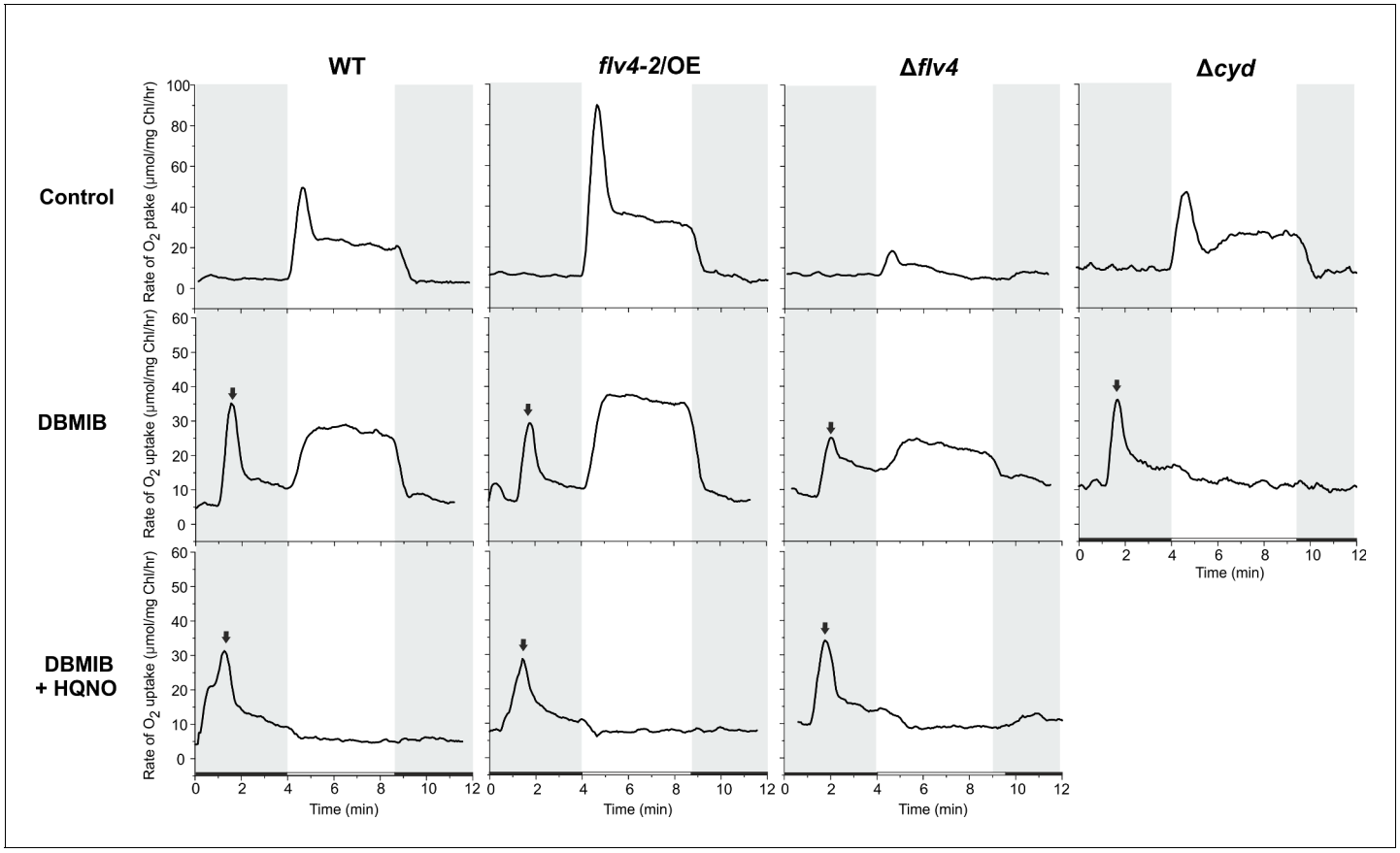

Figure 3—figure supplement 1. O_2 uptake in the WT, *flv4-2*/OE, Δ*flv4* and Δ*cyd* mutant. The cells were grown at LC in BG-11 at pH 7.5. 25 µM DBMIB and 50 µM HQNO were added directly to the cuvette immediately prior to MIMS measurement. The arrow indicates the time when inhibitor was added to the sample. The Δ*cyd* mutant was previously described in ***Howitt and Vermaas (1998)***.

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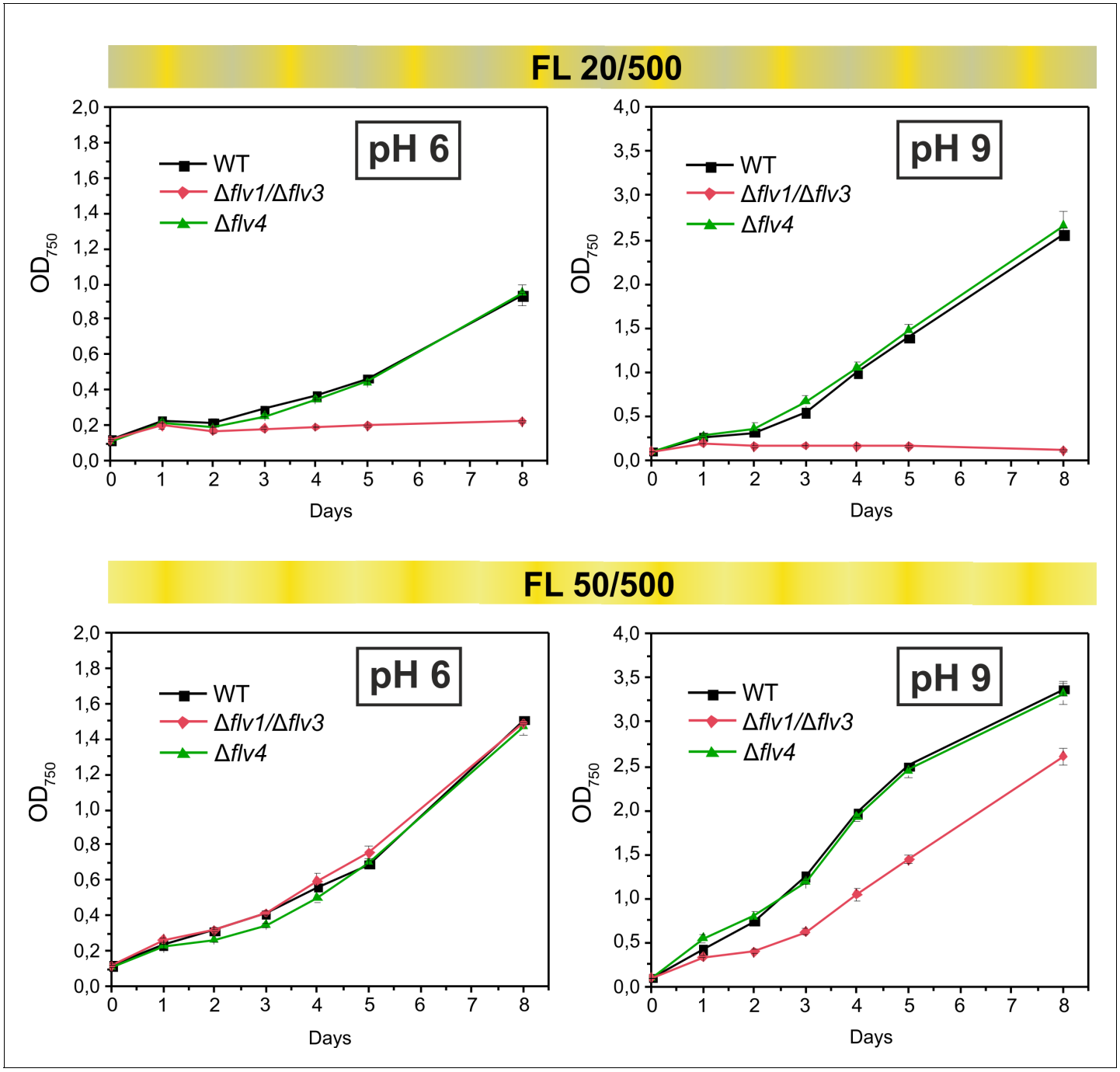

Figure 4. Growth curves of the different FDPs mutants under fluctuating light intensities. Pre-cultures were grown in BG-11 medium under HC for 3 days illuminated with constant light of 50 µmol photons m^{-2} s^{-1}. The cells pre-grown at pH 9 or pH 8.2 (for experimental culture at pH 6) were harvested, resuspended in fresh BG-11 (pH 9 or 6), adjusted to OD_{750} = 0.1 and shifted to LC. Experimental cultures were grown under FL 20/500 or 50/500 regime for 8 days. The experiment was conducted in two independent biological replicates and average values was plotted.

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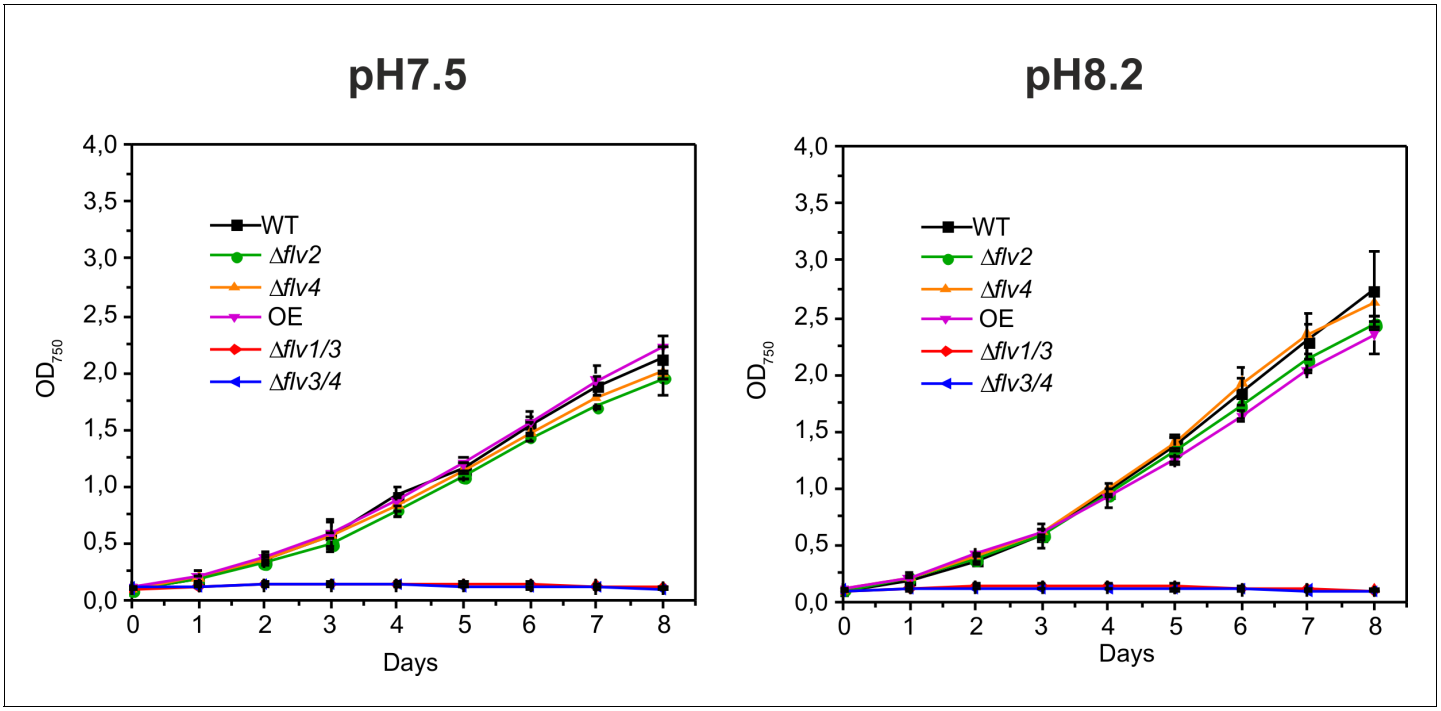

Figure 4—figure supplement 1. Growth curves of the different FDP mutants under fluctuating light intensities (FL20/500 - 20 μmol photons $m^{-2}s^{-1}$ background light is interrupted with 30 s of 500 μmol photons $m^{-2}s^{-1}$ light every 5 min). Cells were grown in BG-11 (pH 7.5) in the absence of Na_2CO_3 and shifted from HC to LC at pH 7.5 or pH 8.2.

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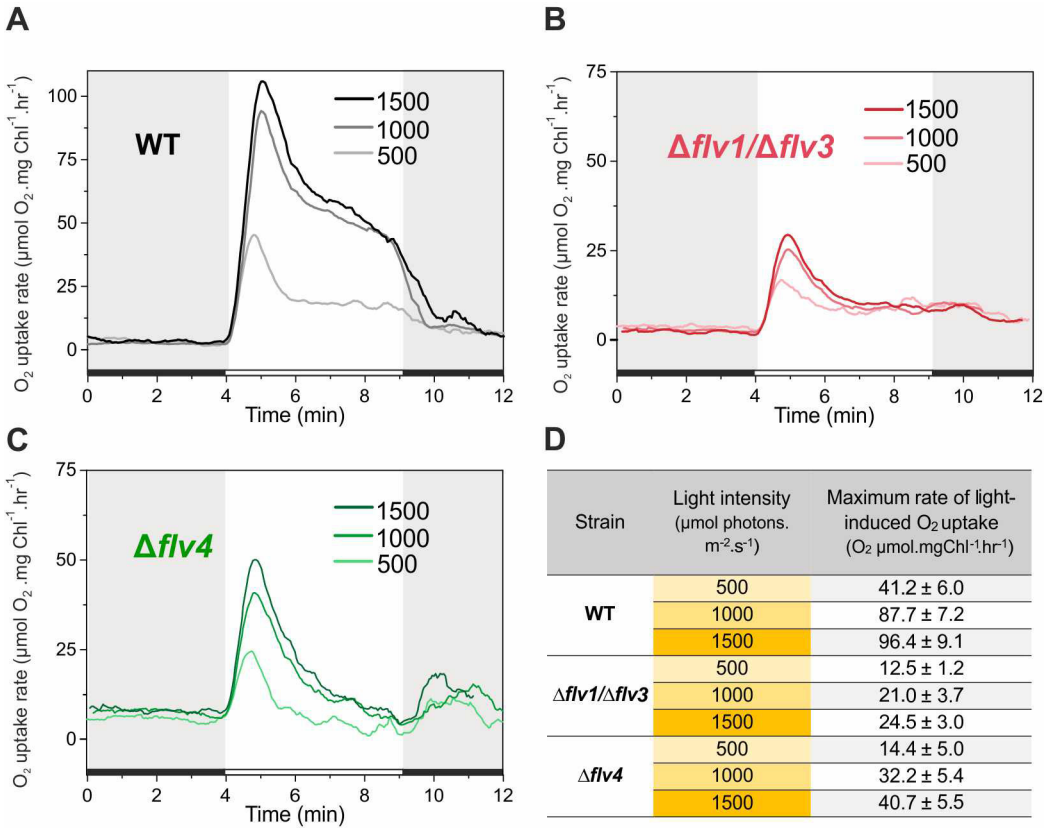

D

Strain	Light intensity (µmol photons. m-2.s-1)	Maximum rate of light-induced O2 uptake (O2 µmol.mgChl-1.hr-1)
WT	500	41.2 ± 6.0
	1000	87.7 ± 7.2
	1500	96.4 ± 9.1
Δflv1/Δflv3	500	12.5 ± 1.2
	1000	21.0 ± 3.7
	1500	24.5 ± 3.0
Δflv4	500	14.4 ± 5.0
	1000	32.2 ± 5.4
	1500	40.7 ± 5.5

Figure 5. Rates of O_2 reduction in response to increasing light intensity in WT, Δ*flv1*/Δ*flv3* and Δ*flv4* mutant cells (A, B, C, respectively). O_2 reduction rate was recorded in darkness (gray background) and under illumination with actinic white light intensities of 500, 1000 and 1500 µmol photons m^{-2} s^{-1} (white background). In order to create comparable conditions for MIMS measurements, all cells were supplemented with 1.5 mM $NaHCO_3$ prior to the measurements. Pre-cultures were grown in BG-11 medium (pH 7.5) under 3% CO_2 (HC) for 3 days and then shifted to LC (atmospheric 0.04% CO_2 in air) at OD750 = 0.2 and pH 7.5 for 4 days. For MIMS measurements, cells were harvested and resuspended in fresh BG-11 medium at a Chl *a* concentration of 10 µg mL^{-1}. (**D**) Maximum rate of light-induced O_2 uptake (O_2 µmol mgChl a^{-1} hr^{-1}) of WT, Δ*flv1*/Δ*flv3* and Δ*flv4* mutant cells at different light intensities applied. The experiment was conducted in three independent biological replicates and a representative plot is shown (***Figure 5—source data 1***).

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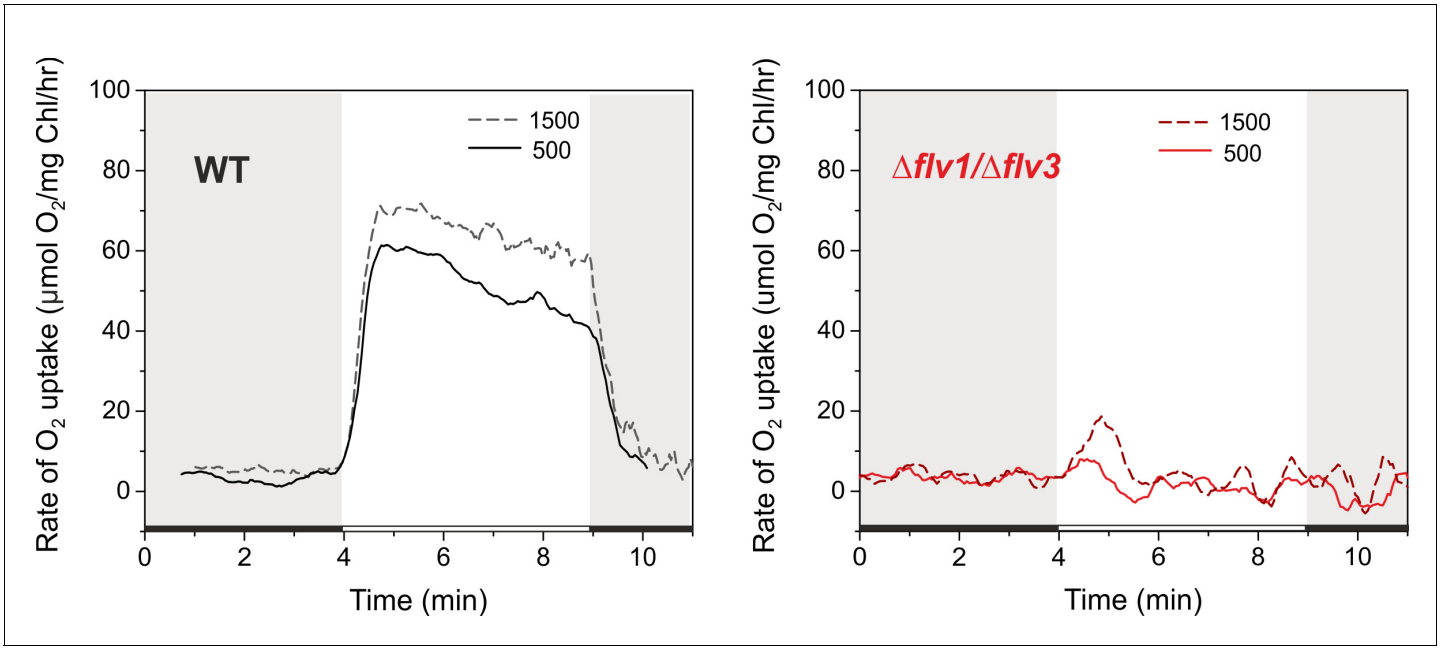

Figure 5—figure supplement 1. Rates of O_2 reduction in response to increasing light intensity in WT and Δ*flv1*/Δ*flv3* mutant cells grown under 3% CO_2 (HC). O_2 reduction rate was recorded in darkness (gray background) and under illumination with actinic white light intensities of 500 and 1500 µmol photons m^{-2} s^{-1} (white background). Cells were grown under 3% CO_2 (BG-11, pH 8.2), harvested and resuspended in fresh BG-11 at Chl*a* 10 ug/ml for MIMS measurements.

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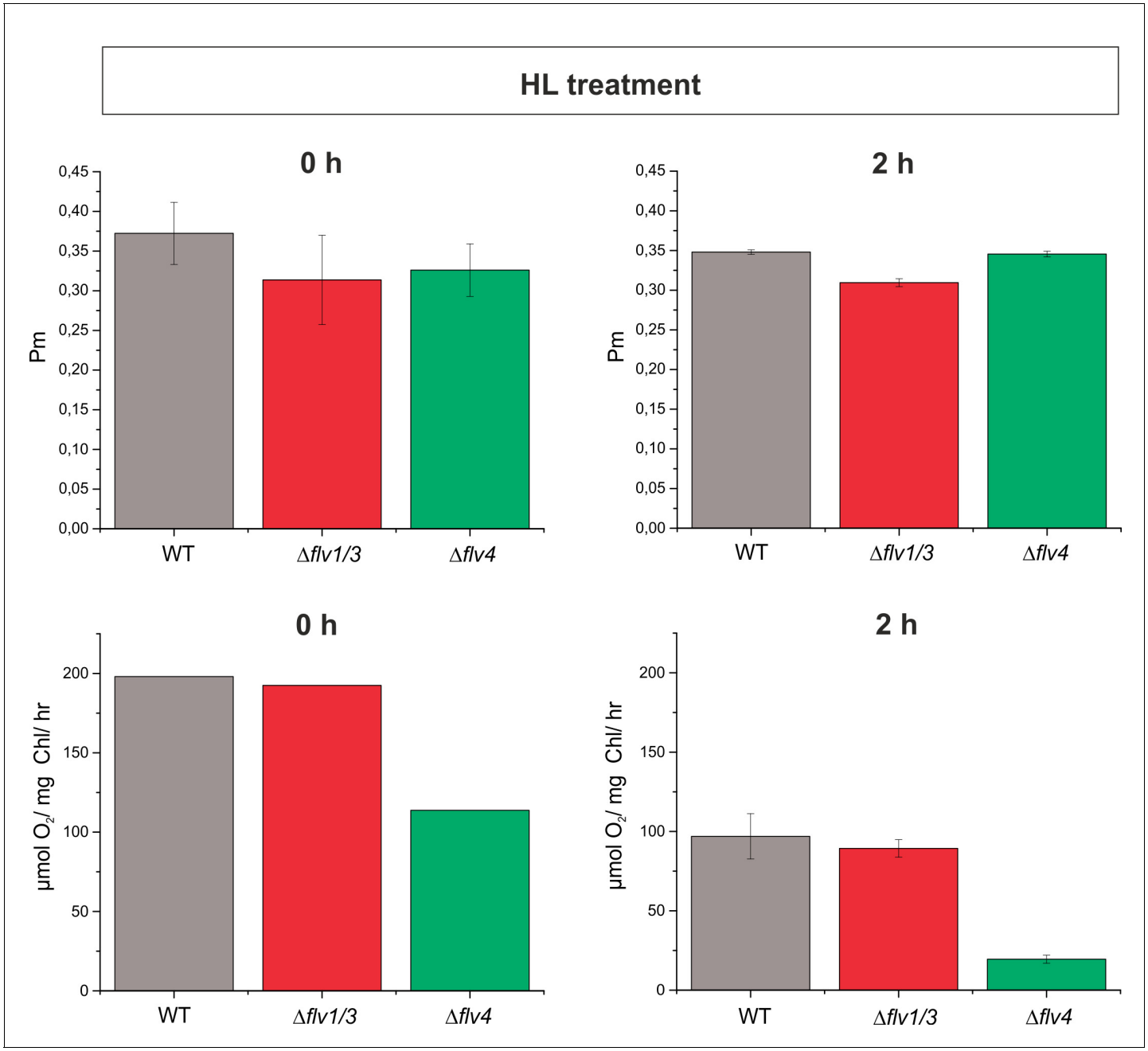

Figure 5—figure supplement 2. The maximum oxidisable amount of P700 (Pm) and PSII activity of the WT, *Δflv1/Δflv3 and Δflv4 mutant cells.* Cells were grown in BG-11 (pH 7.5) and shifted from HC to LC for 4 d and illuminated with 50 µmol photons $m^{-2}s^{-1}$. Prior to the HL treatment, Chl *a* concentration was set to 10 μgmL^{-1}. Measurements were made in dark-adapted samples after 0 hr and 2 hr of HL treatment (1500 µmol photons m^{-2} s^{-1}). The PSII oxygen evolving activity was in measured in the presence of 0.5 mM DMBQ under 1000 µmol photons $m^{-2}s^{-1}$ white illumination. P_m was determined under far red illumination by applying a strong white pulse (5000 µmol photons m^{-2} s^{-1}). Data are represented as mean of 2 biological replicates (± SD).

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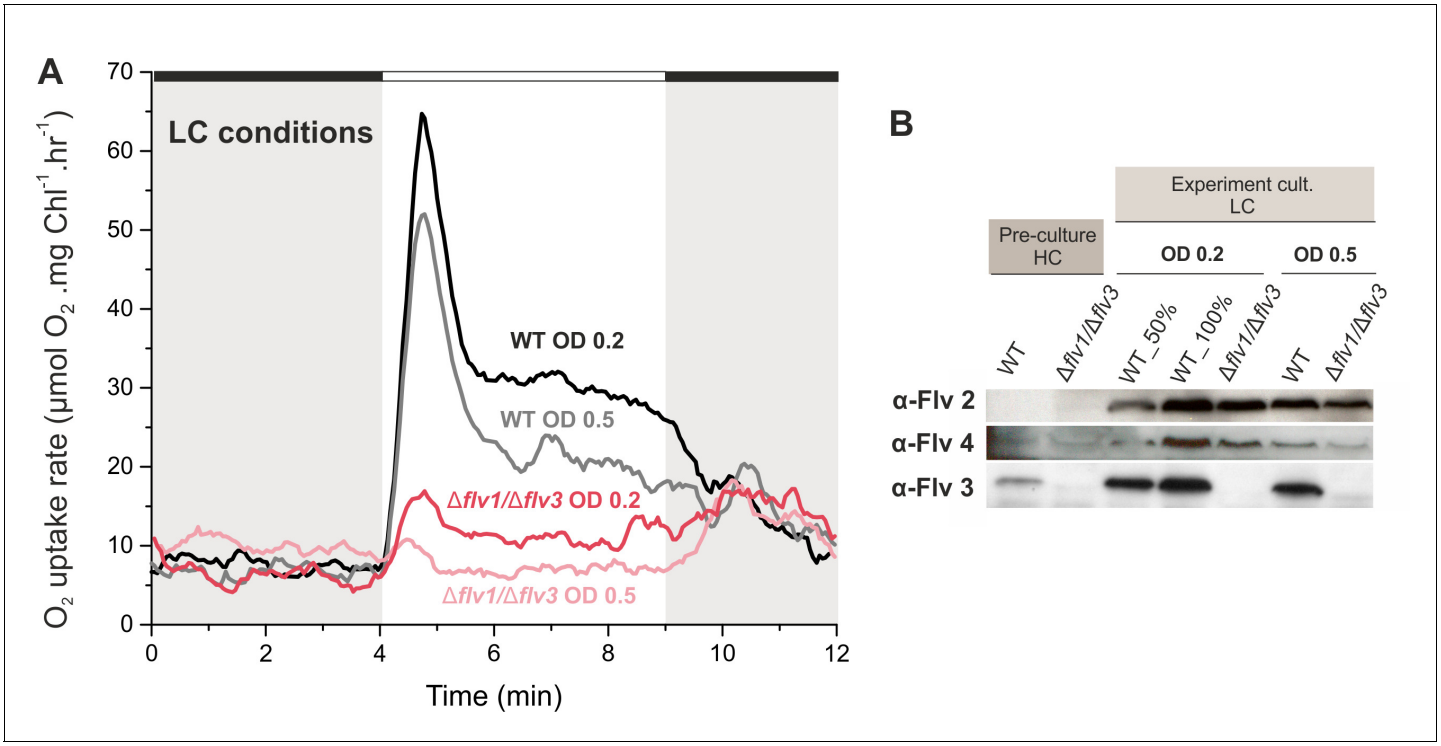

Figure 6. Effect of inoculum size on the O_2 photoreduction and accumulation of FDPs in the WT and Δ*flv1*/Δ*flv3* mutant cells. (**A**) Rates of O_2 uptake measured by MIMS during darkness (gray background) and under illumination with actinic white light at an intensity of 500 µmol photos $m^{-2}s^{-1}$ (white background). In order to create comparable conditions for MIMS measurements, all cells were supplemented with 1.5 mM $NaHCO_3$ prior to the measurements. (**B**) Protein immunoblots showing the relative accumulation of different FDPs in the WT and Δ*flv1*/Δ*flv3* mutant cells. Pre-cultures were grown in BG-11 (pH 8.2) under HC until late logarithmic phase ($OD_{750} \approx 2.5$), then harvested and inoculated in fresh BG-11 under LC at OD_{750} = 0.2 for 4 days or OD_{750} = 0.5 for 3 days. The experiment was conducted in three independent biological replicates and a representative plot is shown in (**A**). WT_50% corresponds to 1:2 diluted total protein sample and 100% to undiluted total protein sample.

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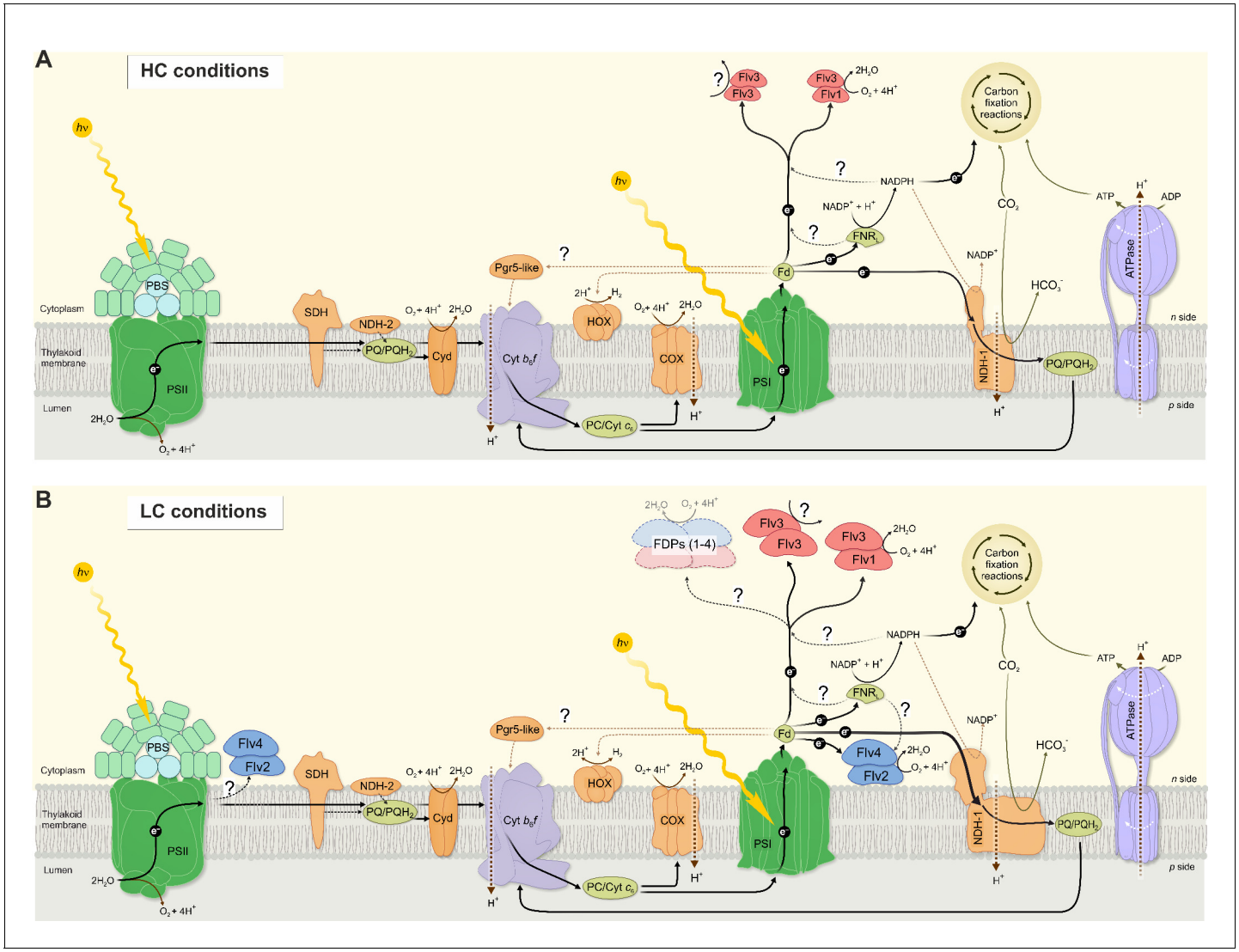

Figure 7. A schematic drawing of photosynthetic light reactions and alternative electron transport routes. (**A**) A steady-state Mehler-like reaction in HC is carried out by the low-abundant, yet catalytically efficient Flv1/Flv3 heterodimer. The Flv3/Flv3 homooligomer is involved in photoprotection as an electron valve with unknown acceptor or as a component of a signaling/regulating network (***Mustila et al., 2016***). (**B**) In LC-grown cells the two pairs of FDP heterodimers are involved in the Mehler-like reaction: Flv1/Flv3 mainly drives rapid and transient O_2 photoreduction and Flv2/Flv4 operates relatively slowly and provides a steady-state background O_2 photoreduction. The soluble Flv1/Flv3 heterodimers function as an immediate acceptor of electrons presumable from reduced Fed, whereas association of Flv2/Flv4 with the thylakoid membrane (and/or Flv1/Flv3) is controlled by *pmf* and Mg^{2+}. Several oligomeric forms of FDPs are hypothesized to exist, including a heterotetramer comprising different FDP protein compositions. The higher abundance of total NDH-1 complexes and FDPs oligomers in LC conditions, compared to HC conditions, is represented by larger size of the protein complexes.

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