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### ► To cite this version:

Pierre Sétif, Ginga Shimakawa, Anja Krieger-Liszkay, Chikahiro Miyake. Identification of the electron donor to flavodiiron proteins in *Synechocystis* sp. PCC 6803 by in vivo spectroscopy. *Biochimica biophysica acta (BBA) - Bioenergetics*, 2020, 1861 (10), pp.148256. 10.1016/j.bbabi.2020.148256 . hal-03032490

**HAL Id: hal-03032490**

**<https://hal.science/hal-03032490>**

Submitted on 30 Nov 2020

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## Identification of the electron donor to flavodiiron proteins in *Synechocystis* sp. PCC 6803 by *in vivo* spectroscopy

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### Abstract

Flavodiiron proteins (FDPs) of photosynthetic organisms play a photoprotective role by reducing oxygen to water and thus avoiding the accumulation of excess electrons on the photosystem I (PSI) acceptor side under stress conditions. In *Synechocystis* sp. PCC 6803 grown under high CO<sub>2</sub>, both FDPs Flv1 and Flv3 are indispensable for oxygen reduction. We performed a detailed *in vivo* kinetic study of wild-type (WT) and  $\Delta flv1/3$  strains of *Synechocystis* using light-induced NADPH fluorescence and near-infrared absorption of iron-sulfur clusters from ferredoxin and the PSI acceptors (F<sub>A</sub>F<sub>B</sub>), collectively named FeS. These measurements were performed under conditions where the Calvin-Benson cycle is inactive or poorly activated. Under such conditions, the NADPH decay following a short illumination decays in parallel in both strains and exhibits a time lag which is correlated to the presence of reduced FeS. On the contrary, reduced FeS decays much faster in WT than in  $\Delta flv1/3$  (13 vs 2 s<sup>-1</sup>). These data unambiguously show that reduced ferredoxin, or possibly reduced F<sub>A</sub>F<sub>B</sub>, is the direct electron donor to the Flv1/Flv3 heterodimer. Evidences for large reduction of (F<sub>A</sub>F<sub>B</sub>) and recombination reactions within PSI were also provided by near-infrared absorption. Mutants lacking either the NDH1-L complex, the homolog of complex I of respiration, or the Pgr5 protein show no difference with WT in the oxidation of reduced FeS following a short illumination. These observations question the participation of a significant cyclic electron flow in cyanobacteria during the first seconds of the induction phase of photosynthesis.

### Introduction

Photosynthetic organisms have developed multiple protective mechanisms to cope with environmental conditions leading to overreduction of the electron-transfer chain, which can be highly deleterious and lead to photoinhibition processes in the presence of oxygen. In cyanobacteria and other oxygenic phototrophs including green algae, mosses and gymnosperms, but not angiosperms, flavodiiron proteins (FDPs) play such a role by oxidizing cytoplasmic/stromal reductants resulting from photosystem I (PSI) activity and reducing oxygen directly to water [1,2]. FDPs are also found in anaerobes where they can reduce either nitric oxide or transiently-present O<sub>2</sub>, protecting them against oxidative stress [3]. FDPs can be grouped in several classes containing different domains [4]. The two N-terminal domains constitute the enzymatic core of all FDPs: a  $\beta$ -lactamase domain containing 2 irons (diiron site) and a FMN-containing flavodoxin domain. Crystallographic structures of bacterial FDPs (see *e.g.* [5,6] revealed that the diiron cluster, the site at which O<sub>2</sub> is reduced, and the FMN moiety of these 2 domains are too far apart ( $\approx 40$  Å) for electron exchange between them and that the minimal functional unit is a head-to-tail dimer which involves one diiron site of a monomer and a close-by FMN from the other one.

Photosynthetic FDPs are composed of 3 domains: from the N to C-terminus, the  $\beta$ -lactamase domain, the flavodoxin domain and a flavin-reductase domain. This last domain binds FMN or FAD, is capable of NAD(P)H

oxidation and has been found to link NADH oxidation to O<sub>2</sub> reduction in a purified FDP protein [7]. The cyanobacterium *Synechocystis* sp. PCC 6803 (thereafter named *Syn.* 6803) contains 4 different FDP genes *flv1* to *flv4* [8]. Whereas the Flv3 and Flv4 proteins have the canonical putative diiron ligands (including acidic residues) similar to those found in FDPs from anaerobes, Flv1 and Flv2 have not and it is still unclear if they can bind iron and reduce O<sub>2</sub> at their presumed enzymatic site [9]. In photosynthetic organisms, FDPs can be grouped in two different phylogenetic clusters, with *Syn.* 6803 Flv1/2 and Flv3/4 belonging to either group. From our present knowledge, FDPs often appear to function in pairs composed of one member of each cluster (Flv1/3 and Flv2/4 in the case *Syn.* 6803) [10,11]. This supports the idea that a FDP functional unit of a photosynthetic organism consists in most cases of a heterodimer composed of monomers of either cluster [11]. In *Syn.* 6803, both Flv2 and Flv4 contribute to O<sub>2</sub> photoreduction when cells are grown under low CO<sub>2</sub> [12,13] whereas it was first found that both Flv1 and Flv3 are indispensable for O<sub>2</sub> photoreduction under high CO<sub>2</sub> conditions [8], as confirmed later on [14]. Whereas the  $\Delta flv1/3$  mutant grows similarly to WT under continuous light and both high and low CO<sub>2</sub>, Flv1 and Flv3 proteins are both necessary for growth under highly-fluctuating light [14,15]. This is attributed to protection by Flv1/3 as a safety valve for excess electrons on the PSI acceptor side, which impedes the formation of ROS species. A similar protective function of FDPs has been documented in all photosynthetic organisms where they were either deleted [16-20] or newly introduced in the case of angiosperms [21-24].

*In vitro* activity studies of photosynthetic FDPs were performed with *Syn.* 6803 proteins in their homooligomeric forms, with homodimers being presumably the minimal functional units. In these studies, NAD(P)H-dependent O<sub>2</sub> reduction was measured, with activities ranging from very small [7] and small [12] to high [25]. These *in vitro* activities are consistent with the presence of a 3<sup>rd</sup> C-terminal domain. In addition, homooligomers of Flv1 and Flv3 were shown to be involved in acclimation of *Syn.* 6803 cells to fluctuating light, in relation with an unknown activity different from O<sub>2</sub> reduction [14].

During photosynthesis, different modes of electron flow can occur: besides the major linear one from water to NADP<sup>+</sup>, alternative electron flows are thought to be involved in regulation, photoprotection and adjustment of the ATP to NADPH ratio. Photosynthetic FDPs are thus the terminal components in a Mehler-like or water to water electron flow. Cyclic electron flow is also considered to be an important pathway, which is thought to involve mostly the NDH-1L complex in cyanobacteria, although another pathway using the protein Pgr5 is still a subject of study [26]. NDH-1L, an analog of complex I of respiration, reduces the plastoquinone pool of photosynthetic membranes by using Fd<sub>red</sub> as electron donor [27,28]. This Fd<sub>red</sub>-oxidation activity of NDH-1L may involve a NDH-1L/PSI supercomplex which has been detected in *Syn.* 6803 [29].

PSI is a light-driven plastocyanin (Pcy)-ferredoxin (Fd) oxidoreductase [30-32]. Pcy and Fd are soluble electron carriers located at either side of the photosynthetic membrane. PSI charge separation leads rapidly to the state P700<sup>+</sup> (F<sub>A</sub> F<sub>B</sub>)<sub>1red</sub> (1red for singly reduced), where P700 is a chlorophyll (Chl.) dimer and (F<sub>A</sub> F<sub>B</sub>) are two [4Fe-4S] clusters in fast redox equilibrium. P700<sup>+</sup> is reduced by Pcy, thus giving Pcy<sub>ox</sub> (oxidized Pcy) on the luminal side of the membrane whereas Fd, containing a [2Fe-2S] cluster, is reduced by (F<sub>A</sub> F<sub>B</sub>)<sub>1red</sub> at the other side. Moreover F<sub>B</sub>, the most exposed PSI cluster, is the electron-transfer partner of Fd. Fd<sub>red</sub> (reduced Fd) has many redox partners, the most important being ferredoxin-NADP<sup>+</sup>-oxidoreductase (FNR) which reduces NADP<sup>+</sup> to NADPH.

Beside the comparison of growth rates and light sensitivities under different conditions, the physiological and functional characterization of FDP deletion mutants was performed, mostly in *Syn.* 6803, by a large variety of methods including gas exchange measurements, gene and protein expression, near-infrared (NIR) P700 absorption and Chl. fluorescence. None of these techniques addresses directly the consumption by FDPs of stromal/cytoplasmic reductants (Fd<sub>red</sub>, NADPH) generated by PSI under light. At present, it is not yet established that NADPH is the electron donor to Flv *in vivo*, although this is suggested by the NAD(P)H-oxidizing activity of purified FDPs from *Syn.* 6803. To investigate this point, we combined in the present work measurements of NADPH fluorescence and Fd absorption in WT and  $\Delta flv1/3$  strains from *Syn.* 6803. Fd measurements were performed with a recently available NIR-transmission spectrophotometer, which allows the measurement of redox changes of Fd together with that of P700 and Pcy [33-35].

## Materials and methods

## Biological materials

WT and mutant strains are from *Syn.* 6803. The  $\Delta flv1/3$  mutant was made as previously described [20]. The  $\Delta ndhD1/D2$  mutant was a gift of Prof. Sonoike (Waseda University, Japan) and was constructed by Ohkawa et al. [36]. The mutant deficient in the gene coding for Pgr5 (*ssr2016*) was made as follows. Part of the gene region was replaced with the kanamycin resistance gene (*Kan<sup>r</sup>*) amplified from the pUC4-KIXX vector [12]. The genomic regions containing *pgr5* were separately amplified by PCR, using gene-specific UP and DN primer sets (UP f: CCGTCCCGGACAAATGGTGGATA; UP r: AAACCGCCCAGTCTACAGTGGAGGGCAATCGCCTTAC; DN f: GTTGGGCTTCGGAATCATCGATGCCAAACAGCGCCA; DN r: GGAGAATGGTTCCACCGAAGG). The three fragments, including UP, *Kan<sup>r</sup>*, and DN were linked by successive PCR to obtain disruption cassettes, and *Syn.* 6803 was transformed using the standard procedure described by Williams [37]. The mutant ( $\Delta pgr5$ ) was selected on BG-11 agar plates that contained kanamycin (20  $\mu\text{g mL}^{-1}$ ). Complete segregation was confirmed using PCR (Supplemental Fig. S11).

WT and mutant strains of *Syn.* 6803 strains were grown at 25°C in BG-11, buffered at pH 7.5 by 20 mM TES-KOH, on a rotary shaker with a light intensity of 150  $\mu\text{moles photons m}^{-2}\text{s}^{-1}$  in 2% CO<sub>2</sub>. For measurements, cells were harvested at a concentration of 6-8  $\mu\text{g Chl./mL}$ , pelleted by centrifugation at 4,000 *g* for 5 minutes and resuspended in fresh BG-11 at a concentration of *c.* 4 and 13  $\mu\text{g Chl./mL}$  for most measurements of NADPH fluorescence and NIR absorption, respectively. Chl. content was measured as previously described [38].

## Spectroscopic measurements

Light-induced NADPH fluorescence measurements were performed with a Dual-PAM spectrophotometer (Walz, Effeltrich, Germany) as previously described [39]. The measuring frequency (MF) was kept at a constant value (100 Hz) and was not increased during illumination, contrary to what was advised [40] and previously done [39], as such an increase was found to result in a small artefactual vertical shift of the signal level, which impedes to measure precisely the duration of lags preceding fluorescence decay at cessation of illumination.

Light-induced redox changes of P700, Pcy and Fd were measured with a DUAL-KLAS-NIR spectrophotometer (Walz, Effeltrich, Germany) as previously described [38]. All measurements were performed at 25°C in the “Slow kinetics mode” at a minimal sampling interval of 1 ms.

For NADPH fluorescence measurements, it is needed to centrifuge cells before resuspending them in a fresh medium (BG-11), because of a strong background fluorescence [40]. As our present work deeply relies on combined measurements of NADPH fluorescence and DUAL-KLAS-NIR absorption, we used for all samples the same centrifugation/resuspension treatment, also followed in most cases by dark adaptation for at least 8 mins, for both types of measurements.

## DUAL-KLAS-NIR preliminary and control measurements

Deconvolution of the DUAL-KLAS-NIR transmission signals requires the knowledge of the spectral shapes (which will hereafter be named DMPs, *i.e.* differential model plots) of the 3 species to be deconvoluted, *i.e.* P700, Pcy and Fd. It has been shown in a recent *in vitro* study that the terminal acceptors ( $F_A F_B$ ) of PSI give a DMP similar to that of Fd [41]. Moreover it has also been shown that, if Fd and ( $F_A F_B$ ) are both reduced by illumination, their signals could be measured together in an additive way. As our results will show that ( $F_A F_B$ ) are indeed reduced during our measurements, the so-called Fd contribution will be renamed “FeS” in the present work, in order to underline that it may arise from both the [2Fe-2S] cluster of Fd and the [4Fe-4S] clusters of PSI.

Procedures involving different light/dark (pre)treatments have been proposed to measure *in vivo* the individual DMPs of P700, Pcy and Fd/FeS [33]. These recipes rely on the possibility to get a signal change which is selective for one species while the two others give negligible contributions. For Fd/FeS, the published procedure (Fig. 6 in [33]) was used with only a change in the illumination time (300-400 ms). Measuring the P700 and Pcy DMPs (Fig. 7 and 8 in [33], respectively) requires an adaptation of the procedures, due to the fact that the PSI/PSII ratio is much larger in *Syn.* 6803 ( $\geq 3$ ) than in plants ( $\approx 1$ ). To compensate for the small relative amount of PSII, it was necessary for both types of measurements to replace the single turnover flash

by a series of flashes or a longer high-intensity illumination in order to increase the amount of PSII-originating electrons driving  $P700^+$  and  $Pcy_{ox}$  reduction. For both types of measurements (P700 and Pcy), either 3 to 6 single turnover (ST) flashes separated by 1 ms time intervals or a single MT flash of *c.* 5 ms was found to allow pure species to be measured. The *Syn.* 6803 DMPs measured *in vivo* and used in the present study are given in Fig. SI2. All DMPs were measured with the same WT strain (+ the  $\Delta flv1/3$  mutant in Kobe) using 2 different DUAL-KLAS-NIR spectrophotometers located either in Kobe University or in Saclay laboratory. For each chemical species, its DMPs differ somewhat when measured in one laboratory or another. This should be related to the fact that the LEDs emission spectra slightly differ from one spectrophotometer to another (Dr. C. Klughammer, personal communication).

A procedure allowing determination of the 100% amplitudes of P700, Pcy and FeS signals has also been described [34]. This procedure was adapted to measure these amplitudes in the different strains by reference to an amplitude of 1 for P700.

The relative absorption coefficients of P700, Pcy, Fd and  $(F_A F_B)$  at 902.5 nm were previously determined *in vitro* for tobacco and *T. elong.* [41]. These values can also be calculated for *Syn.* 6803 from data in this last paper and are given in Table 1. In the description of some of the results, it will be assumed that these values can be extrapolated to *in vivo* measurements, as supported by the fact that quasi-ideal conditions of light transmission appear to be met with *Syn.* 6803 cells.

Methyl viologen (MV) at 300  $\mu$ M is known to oxidize  $Fd_{red}$  and  $(F_A F_B)_{red}$  *in vitro* in the millisecond timerange and it should inhibit the stationary formation of both reduced species *in vivo* [42]. Fig. SI3 shows the effect of MV addition on *in vivo* signals of P700, Pcy and FeS, with signals shown in percent of their maximal amplitude. Whereas the transient reduction of  $P700^+$  is inhibited by MV addition and the Pcy signal is little modified, the FeS signal has considerably decreased 7-9 minutes after MV addition and has completely disappeared 10 minutes after. This gives evidence that FeS are indeed correctly identified and measured through deconvolution of NIR signals.

## Results

### NADPH fluorescence of WT and $\Delta flv1/3$

Since NADPH has been proposed to be the electron donor to FDPs, first we measured the light-induced kinetics of NADPH fluorescence in WT and  $\Delta flv1/3$ . Such fluorescence kinetics were previously studied in detail in WT [39]. Whereas NADH can also contribute to the signals as its fluorescence is identical to that of NADPH, the kinetic properties of the light-induced signals, such as the fast rise at light onset and the light activation of decay, indicate that the most part of the light-dependent fluorescence signals can be attributed to NADPH. In the following, we will assume that this is indeed the case. The fluorescence kinetics were compared under different conditions of continuous illumination. Identical kinetics are observed for the fluorescence rise at the onset of illumination (Fig. 1, Fig. SI4) and the fluorescence decay after a prolonged illumination, when the Calvin-Benson cycle (CBc) is activated (Fig. 1/D). The similarity in rising kinetics suggests that the FNR content of both strains is similar. After prolonged illumination, the similarity in decay kinetics shows that the presence of Flv1/3 has no observable effect on NADPH decay, when the activated CBc drives a high NADPH consumption.

NADPH kinetics were also studied with a short illumination period of 400 ms (Fig. 1/ABC). This duration was chosen as it is long enough to elicit a full fluorescence rise whereas it only weakly activates the CBc [39]. Moreover signal averaging was performed at a time interval of 1 min., which should allow significant CBc deactivation between 2 consecutive measurements (note however that the signal undershoot observed at  $t > 4-5$  s in Fig. 1/A is diagnostic of the activation of a NADPH consuming enzyme, corresponding presumably to partial CBc activation). Under these conditions, the fluorescence decay is, as expected, slower than after CBc activation. Thus the time needed for 50% signal decay is 2.2 and 2.65 s for WT and  $\Delta flv1/3$ , respectively, whereas, after 120 s illumination, this time drops to 0.38 and 0.36 s for WT and  $\Delta flv1/3$ , respectively.

For evaluating the Flv1/3 involvement in NADPH oxidation, it is required to compare post-illumination decay kinetics without any type of signal normalization. This condition is almost met in Fig. 1 as the  $\Delta flv1/3$  signal

was multiplied by a small factor of 1.04 in order to normalize the light-induced signal allowing us to compare more easily the rising and decay kinetics. This indicates that the NADP photoreducible pools are quite similar in WT and  $\Delta flv1/3$ . We repeatedly compared the NADP-reducible pool sizes in WT and  $\Delta flv1/3$  and found values comprised between 1.03 and 1.15 for the  $\Delta flv1/3$  to WT pool ratio. The similarity in NADP pool sizes is also shown in Fig. S15, in which NADPH fluorescence is measured at different chlorophyll concentrations.

The longer 50% signal decay time in  $\Delta flv1/3$  after 400 ms illumination seems at first sight to be in accordance with NADPH oxidation by Flv1/3. However this is misleading as it can be seen that fluorescence decays almost in parallel in both strains (Fig. 1/A) and the most conspicuous difference in kinetics is the presence of a lag preceding the decay in  $\Delta flv1/3$  (Fig. 1/B). This decay is c. 700 ms long whereas it was clearly visible in WT only after extensive averaging, with a value of 140 ms (Fig. 1/C). It can also be noted that the lag decreases after CBc activation as it was not any longer observed in both strains after 120 s illumination (Fig. 1/D).

The origin of these lags is easily interpreted if one assumes that stromal reductants besides NADPH, *i.e.*  $Fd_{red}$  and reduced PSI acceptors (collectively named  $FeS_{red}$  in the following), are accumulated in the light and that, after light cessation, these reductants are consumed via FNR catalysis to maintain the NADP pool at its maximum state of reduction. The lag would then correspond to the time it takes for these reductants to be reoxidized. This hypothesis will be tested below by measuring the kinetics of  $FeS_{red}$  reoxidation after illumination.

### DUAL-KLAS-NIR kinetics of P700, Pcy and FeS in WT and $\Delta Flv1/3$

Kinetics of P700, Pcy and FeS redox changes were first measured in a procedure allowing us to obtain the maximum amplitudes of the 3 species (P700, Pcy and FeS) [34] (Fig. 2). When WT and  $\Delta flv1/3$  kinetics were compared in detail, the following was observed:

1) Following dark adaptation, the P700 kinetics at the onset of actinic light are initially similar in both strains, with a fast rise followed by a fast decay during the first 300-400 ms (Fig. 3). Such observations have already been reported and interpreted as follows [15,43,44]: the initial rise and decay are attributed to PSI charge separation and stabilization followed by reduction by incoming electrons due to PSII activity. After this initial increase/decay pattern, the P700 kinetics strongly differ in WT and  $\Delta flv1/3$ , as P700 remains reduced in  $\Delta flv1/3$  whereas P700<sup>+</sup> increases to a large level in WT. This large WT level involves a donor-side limitation that has been shown to protect PSI from photooxidative damage [20,45]. This protection involves electron transfer to O<sub>2</sub> catalyzed by Flv1/3. In  $\Delta flv1/3$ , where this flow is absent, the high reduction level of the stromal electron acceptors limits P700 photooxidation, an effect which may involve charge recombination reactions (PSI acceptor-side limitation) or cyclic electron flow [43].

2) The Pcy kinetics follow the P700 kinetics with little Pcy<sub>ox</sub> accumulated in  $\Delta flv1/3$  after 400 ms.

3) The FeS reduction kinetics are quite similar in WT and  $\Delta flv1/3$  at the onset of actinic light but deviate after 500 ms. Whereas a high level of  $FeS_{red}$  remains during illumination in  $\Delta flv1/3$  (signal between 3 and 6 s in Fig. 2/B),  $FeS_{red}$  decays in WT, an effect which can be attributed to the fact that P700 becomes almost entirely oxidized during this period. By applying a MT pulse of high intensity at 350 ms, the maximal signal of Fd reduction was claimed to be measurable [34], but this maximal signal will be discussed in detail further below.

The maximal signals due to P700 and Pcy oxidations (Table 2) are obtained by subsequent application of a long MT pulse which is given at the end of a far-red illumination period (10 to 20 s in Fig. 2; [34]). From the chlorophyll content of the samples and the *in vitro* determination of the P700<sup>+</sup> coefficient at 902.5 nm with isolated PSI from *Syn. 6803* [41], theoretical P700<sup>+</sup> signals can also be calculated together with amplification factors.

An amplification factor of 1 would be consistent with ideal optical properties with an optical pathlength corresponding to the cuvette width, *i.e.* with no contribution from light refraction and reflexion within the cells. The fact that these factors are just above 1 for both *Syn. 6803* strains is in accordance with recent measurements [38] and indicates that the present measurements are made under quasi-ideal conditions of light transmission. In turn, this provides support to the idea that the relative coefficients measured *in vitro* (Table 1) can be extrapolated to *in vivo* conditions in order to estimate the stoichiometries of the different

electron-transfer components. This property was used to calculate Pcy/PSI ratios of 2.2-2.3 in both strains (Table 2).

### Kinetics of FeS<sub>red</sub> reoxidation after a short illumination in WT and $\Delta flv1/3$

DUAL-KLAS-NIR kinetics were further studied with the same samples as above using extensive averaging of signals induced by actinic red light of high intensity (8100  $\mu\text{moles photons m}^{-2}\text{s}^{-1}$ ) and 350 ms duration (Fig. 3, Fig. 4/traces b and d). This duration is optimal for 3 reasons, *i.e.* just completed NADP<sup>+</sup> photoreduction (Fig. 1/B), limited activation of the CBc and minimal extent of P700/Pcy photooxidation. Moreover, the difference in P700<sup>+</sup> and Pcy<sub>ox</sub> levels between WT and  $\Delta flv1/3$  are relatively small at the end of illumination, so that the subsequent dark FeS<sub>red</sub> decays can be compared with minimal interferences from recombination reactions and imperfect 3-species deconvolutions. A significant level (35%) of P700 oxidation is though observed in WT, contrary to  $\Delta flv1/3$ . However, at the end of illumination, the P700 signal decays much faster than the FeS signal. This implies that no or very little recombination is involved in the WT FeS<sub>red</sub> decay. From Figs. 3 and 4, it can be seen that FeS<sub>red</sub> decays much more slowly in  $\Delta flv1/3$  than in WT whereas light-induced FeS reductions are quite similar. Similar FeS<sub>red</sub> decay kinetics were also measured after illumination with a light intensity similar to that used for NADP<sup>+</sup> photoreduction (traces a and c in Fig. 4). Monoexponential fits of Fd<sub>red</sub> give values 13.1-13.3 s<sup>-1</sup> and 2.3-2.4 s<sup>-1</sup> in WT and  $\Delta flv1/3$ , respectively. This difference in FeS decay kinetics between WT and  $\Delta flv1/3$  fits nicely with the different lags that were observed for NADPH decay in the 2 strains.

These observations confirm our hypothesis that the lags observed in NADPH decays correspond to consumption of FeS<sub>red</sub> by FNR, which maintains the NADP pool at its fully reduced level as long as some FeS<sub>red</sub> is available. Moreover, and more importantly, the difference in FeS<sub>red</sub> oxidation kinetics between WT and  $\Delta flv1/3$  in combination with the slower and identical NADPH decays (disregarding the lags) shows that Flv1/3 accept electrons upstream from NADPH, at the FeS<sub>red</sub> level.

### Evidence for recombination between P700<sup>+</sup> and F<sub>xred</sub>. Contribution of Fd to the NIR FeS signal

Schreiber and Klughammer [34] published a procedure for measuring the maximal NIR signal of Fd reduction by applying an intense MT pulse superimposed on a high intensity actinic light under conditions where the CBc is not activated, so that Fd<sub>red</sub> reoxidation is relatively slow (Fig. 2). This determination implicitly relies on the idea that Fd<sub>red</sub> can be fully accumulated without NIR contribution due to reduction of the [4Fe-4S] terminal PSI acceptors. However *in vitro* measurements recently showed that (F<sub>A</sub> F<sub>B</sub>) reduction can contribute to NIR signals [41]. It is also expected that reduction of F<sub>x</sub>, the [4Fe-4S] preceding (F<sub>A</sub> F<sub>B</sub>) in PSI, should give as well a NIR contribution. Moreover, if PSI [4Fe-4S] acceptors are reduced, they could be involved in subms to 100 ms recombination reactions, so that their stationary reduction state may highly depend on the light intensity and be possibly incomplete. To examine this matter, we extensively averaged signals under the above conditions to check whether we can obtain evidence for recombination reactions. A sample similar to that studied above was submitted to high intensity red light for 1 s to which a 100 ms MT pulse of very high intensity was superimposed between 300 and 400 ms after the onset of actinic light. The measurements were made at a low repetition rate in order to minimize CBc activation. The WT results shown in Fig. 5A, where P700, Pcy and FeS signals are plotted with Y-scales proportional to their relative differential absorption coefficients (Table 1), suggest that recombination occurs between P700<sup>+</sup> and F<sub>xred</sub> at the end of the MT pulse (t = 0.4 s). This is seen as an unresolved step signal (sampling time is 1 ms), which exhibits a signal ratio FeS/P700 of -0.079. This ratio is 1.8 times larger than the (F<sub>A</sub> F<sub>B</sub>)<sub>single red</sub>/P700 ratio of -0.044 (Table 1), a difference which may be attributed either to imperfect deconvolution or to a NIR differential absorption of F<sub>x</sub> larger than that corresponding to single reduction of (F<sub>A</sub> F<sub>B</sub>). The same measurements were performed with  $\Delta flv1/3$  (Fig. 5B) with results qualitatively similar to those of WT, but with a smaller amplitude of both P700 and FeS signals that can be attributed to fast recombination between P700<sup>+</sup> and FeS<sub>red</sub>. The reason why the signals attributed to recombination are smaller in  $\Delta flv1/3$  than in WT may be that some F<sub>xred</sub> is partially accumulated in  $\Delta flv1/3$  before the MT pulse. This interpretation is in accordance with the larger acceptor side limitation in this strain.

Assuming the above conclusion about  $F_x$  involvement in NIR absorption, the maximal FeS signal observed in Figs. 3 and 4 should then correspond to both Fd and ( $F_A F_B$ ) fully reduced. In turn, by using the relative absorption coefficients measured *in vitro* (Table 1), the ( $F_A F_B$ ) contribution was estimated to 71-74% of this maximal signal in both WT and  $\Delta Flv1/3$  whereas the minor Fd signal would correspond to a Fd/PSI ratio of 1.1-1.4, in agreement with [46]. There is quite evidently a large uncertainty in this last estimation and more work is needed to derive the Fd/PSI ratio in cyanobacteria from NIR measurements. However we would like to emphasize here that the FeS signal most probably includes a large, and possibly major, contribution of ( $F_A F_B$ ) reduction.

### **FeS accumulates more rapidly when the NADP is highly reduced**

Experiments shown in Fig. 6A/B are similar to those of Fig. 4, except for a lower intensity and a longer, but still short, illumination with actinic light. Moreover, the initial FeS kinetics (traces a) were compared to those induced by a second illumination given after a delay just sufficient for  $FeS_{red}$  reoxidation (traces b; delay between the end of 1<sup>st</sup> and beginning of 2<sup>nd</sup> = 0.4 and 0.9 s for WT and  $\Delta flv1/3$ , respectively). These delays are short enough to allow NADPH to be still almost entirely reduced at the beginning of the 2<sup>nd</sup> illumination (Fig. 1). For both strains,  $FeS_{red}$  reduction is faster during the 2<sup>nd</sup> illumination since the sigmoidal pattern occurring around 100 ms during the 1<sup>st</sup> illumination is no longer present. These data can be simply interpreted by assuming that sigmoidicity arises from fast  $Fd_{red}$  consumption by FNR which occurs as long as  $NADP^+$  is not fully reduced. This interpretation also fits with the kinetics of NADPH fluorescence rise as, during 1<sup>st</sup> hv measurements, the FeS reduction resumes (or the lag ends) at c. 100 ms, a time at which most of NADPH is formed (Fig. 1B). When the delay separating the two illumination periods is long enough for large reoxidation of NADPH, the  $FeS_{red}$  kinetics (traces c) are back to those obtained at the 1<sup>st</sup> illumination. These kinetic changes are also displayed in Fig. 6C by plotting the signal at 100 ms after the beginning of the 2<sup>nd</sup> illumination as a function of the delay between the illuminations. The presence of a lag in both strains is consistent with a FNR reduction process being dominant as long as the NADP pool is not fully reduced, regardless whether Flv1/3 is present or not. The kinetics following the lag are also rather similar in WT and  $\Delta flv1/3$  (see also Fig. 3). This indicates that Flv1/3 has only a moderate effect on  $FeS_{red}$  accumulation under the present conditions of illumination. In turn, this suggests that  $FeS_{red}$  accumulation is dictated by reactions faster than reoxidation by Flv1/3, namely donation to  $P700^+$  and recombination reactions between  $P700^+$  and PSI acceptors and competition between these two processes.

The same measurements were performed with a mutant in which the *pgr5*-like gene was deleted. NIR kinetics in the mutant were virtually identical to those in WT (Fig. 7A) as is also the case for the dependence of the 100-ms signal size upon the delay between the two illuminations (Fig. 7C). This result is consistent with the fact that no significant effect of Pgr5 deletion was previously observed in cyanobacteria [15], although Pgr5 was proposed to be involved in cyclic electron transfer in *Syn. 6803* [26,47].

### **The $FeS_{red}$ decay kinetics in the absence of NDH-1L are very similar to those of WT**

In a mutant with deleted *ndhD1* and *ndhD2* genes, in which the NDH-1L complex is expected to be absent [48], reoxidation of  $FeS_{red}$  after a short illumination was found to be quite similar to those of WT (Fig. 7B). In darkness, the NADP pool was found to be almost entirely reduced in this mutant (Fig. 7D): Illumination of a dark-adapted sample leads only to a small signal increase followed by a similarly small extent of decay (Fig. 7D, trace a). By contrast, a prolonged illumination is followed by a decay of larger amplitude and faster kinetics. Noticeably, the decay kinetics after prolonged illumination are similar to those of WT and  $\Delta flv1/3$  (Fig. 1D), which is attributed to CBc activation in all cases. These data also show that the photoreducible NADP pool is almost entirely reduced in the  $\Delta ndhD1/D2$  mutant in darkness. In accordance with this highly reduced state, the 1<sup>st</sup>-illumination FeS reduction kinetics (Fig. 7B, trace a) exhibits almost no sigmoidicity and are similar to those of the 2<sup>nd</sup>-illumination kinetics (Fig. 7B, trace b) as it is also shown from the similarity in the 100-ms signal size for the 2 corresponding illuminations (Fig. 7C). In summary, our observations are in accordance with the fact that the NADP pool remains essentially reduced in  $\Delta NdhD1/D2$  as long as the CBc is not activated and support our interpretation for the 1<sup>st</sup>-illumination lag observed in the other strains.

## Discussion

### NIR signals as a tool to measure reduction of Fd and iron-sulfur clusters of PSI

The possibility of recording *in vivo* Fd reduction by real-time NIR spectroscopy has been developed recently [33-35]. This measurement results from deconvolution of NIR signals into contributions of P700, Pcy and Fd. From an *in vitro* NIR study, it has been later determined [41]: 1) that reduction of PSI iron-sulfur clusters is measured together with Fd reduction 2) that cytochrome  $c_6$  oxidation (when present instead of Pcy), and by extrapolation probably also cyt.  $f$  oxidation, can contribute to NIR signals and 3) that P700 and Pcy oxidations give much larger signals than those attributed to reduction of Fd and PSI clusters. This underlines the difficulty of specifically measuring the kinetics of FeS (collective name for Fd,  $F_A$  and  $F_B$ ) reduction by this method. In the present work, we provide several lines of evidence which show that FeS reduction and reoxidation can indeed be reliably measured in *Syn. 6803* cells: 1) the FeS signal is no longer observed after incubation with MV (Fig. SI3); 2) the initial FeS reduction kinetics strongly depend on the redox state of the NADP pool at the onset of illumination (Figs. 6 & 7); 3) the  $FeS_{red}$  reoxidation kinetics following a short illumination are different in WT and  $\Delta flv1/3$  (Figs. 3-4), contrary to those of  $P700^+$  and  $Pcy_{ox}$  reduction kinetics which are quite similar (Fig. 3), and these reoxidation kinetics are consistent with the variable NADPH lag observed in both strains (Fig. 1). The global internal consistency of our data provides strong support to the idea that FeS reduction can indeed be measured, even though one cannot exclude that a minor part of the signals attributed to FeS results from imperfect deconvolution or from contribution of other species such as cyt.  $f$ . In this respect, it should be noted that Pcy is largely expressed in our conditions of cell growth so that we expect little, if any, cyt.  $c_6$  contribution.

### Fd<sub>red</sub> (or F<sub>Bred</sub>), and not NADPH, is the electron donor to Flv1/3

The presence of both Flv1 and Flv3 proteins is indispensable for oxygen photoreduction in *Syn. 6803* under high-CO<sub>2</sub> conditions [8] which strongly suggests, but does not prove, that the Flv1/3 heterodimer is responsible for this activity. In the following, we will implicitly assume that this is the case. We compared high-CO<sub>2</sub> grown *Syn. 6803* WT and  $\Delta flv1/3$  by a combination of 2 different *in vivo* spectroscopic probes, NADPH fluorescence and NIR absorption decrease due to FeS reduction. Most measurements were performed under specific conditions expected to enhance the difference between WT and  $\Delta flv1/3$ , where the PSI acceptor side is highly photoreduced at the end of the illumination period whereas the CBc is weakly activated. This allowed us to conclude that Fd<sub>red</sub>, or possibly the terminal acceptor F<sub>Bred</sub> of PSI, and not NADPH, is the redox partner of Flv1/3. The possibility that Fd is an interacting partner of Flv proteins has been previously proposed from Fd-chromatography in *Syn. 6803* [49], from a 2-hybrid assay study in *Chlamydomonas reinhardtii* [50] and from a recent study showing that, after a short illumination period, Fd<sub>red</sub> is reoxidized more slowly in the absence of Flv3 [51].

The uncertainty about the exact nature of the Flv1/3 partner (Fd<sub>red</sub> or F<sub>Bred</sub>) derives firstly from the fact that the reduction of Fd and of the PSI [4Fe-4S] clusters cannot be spectrally distinguished and are measured in an additive manner and secondly from the observation that Fd,  $F_A$  and  $F_B$  appear to be all photoreduced during our measurements (Fig. 3 and 4), as deduced by the likely observation of a recombination reaction between  $P700^+$  and  $F_{xred}$ , when a very intense pulse light is superimposed to the light period (Fig. 5). If F<sub>Bred</sub> is the partner of Flv1/3, then Fd<sub>red</sub> oxidation by Flv1/3 could also proceed indirectly via uphill population of F<sub>Bred</sub>, so that it should be slower than F<sub>Bred</sub> oxidation itself, and *a fortiori* slower than ( $F_{Ared}$ , F<sub>Bred</sub>) oxidation. In turn, the decay of  $FeS_{red}$  in WT should be multiexponential (Fig. SI6/E), which is not seen in the fits of Fig. 4A. However we estimate that the present signal-to-noise ratio of our measurements of  $FeS_{red}$ , together with the uncertainties in the precise deconvolution of the NIR signals, does not allow us to decide for the absence or presence of several exponential components. Thus we cannot definitely conclude from the shape of the  $FeS_{red}$  decay whether Fd<sub>red</sub> or F<sub>Bred</sub> is the Flv1/3 partner. It is also noticeable that the possibility that F<sub>Bred</sub> is the partner of Flv1/3 is related to the *in vivo* ratio of Flv1/3 to PSI, as a small ratio seems to be incompatible with an efficient oxidation of F<sub>Bred</sub> by Flv1/3. To our knowledge, this ratio has yet not been determined.

The fact that NADPH is not the main electron donor to Flv1/3 appears surprising in view of the presence in Flv1 and Flv3 of C-terminal domains which are expected to have a NAD(P)H-oxidizing activity, as supported by *in vitro* measurements of purified proteins [7,12,25]. Moreover, the absence *in vivo* of significant Flv1/3-

dependent NADPH-consuming activity, as deduced from our measurements, seems to contradict some recent results [25]. In this paper, NADPH oxidation turnover numbers 25 and 32 s<sup>-1</sup> were measured under atmospheric levels of O<sub>2</sub> for Flv1 and Flv3, respectively, and similar activities were measured with NADH. In the case of Flv3, biochemical evidence shows that the Flv3 dimer is the functional unit, and it is also most probably the case for Flv1, as supported by cooperativity measurements for both NAD(P)H and O<sub>2</sub> binding [25].

These high activities of Flv1 and Flv3 dimers contradict previous observations that both Flv1 and Flv3 are indispensable for oxygen photoreduction in *Syn. 6803* [8]. These observations are in accordance with the strong phenotypes resulting from single deletion of either *flv1* or *flv3* [15] or their homologs in other photosynthetic organisms [18,19,52]. It is noticeable that a straightforward interpretation of single deletion mutants is in general not possible as deletion of one gene of a pair decreases or abolishes expression of the other one [15,18]. Nevertheless, one rather clear case has been reported: in the *flv1* deletion mutant of *Syn. 6803*, where oxygen photoreduction is absent, Flv3 is still expressed at a decreased but significant level [15]. This indicates that the Flv3 dimer is probably not active in oxygen reduction in this organism, as also suggested in [14]. The discrepancy with the latest *in vitro* results [25] requires further investigation.

Roles other than NAD(P)H oxidation may be hypothesized for the Flv1/3 C-terminal domains:

1) NAD(P)H oxidation by the flavins of these domains may indeed occur *in vivo* but at a much slower rate than FeS<sub>red</sub> oxidation. This reaction may also occur in the other direction (see 3).

2) The C-terminal domains may be involved in Fd binding, only by themselves or concomitantly with the flavodoxin domains. The most simple electron transfer pathway would then be linear : FeS<sub>red</sub> → Flavin<sub>C-terDom</sub> → FMN<sub>flavodoxin</sub> → diiron.

3) Alternately, the electron transfer pathway may be branched with FeS<sub>red</sub> reducing the flavodoxin FMN: FeS<sub>red</sub> → FMN<sub>flavodoxin</sub> → diiron, and the flavodoxin FMN being a branching point involved in the reaction: FMN<sub>flavodoxin</sub> → Flavin<sub>C-terDom</sub> → NAD(P)<sup>+</sup>. During this reaction, the role of the C-terminal domains would be to store electrons, or even to reduce NAD(P)<sup>+</sup>. Such reactions could become significant in case of enzyme over-reduction, *i.e.* when the pathway to diiron is slowed down, *e.g.* in low O<sub>2</sub>.

The question could be asked whether NADPH decay via FNR-catalysed up-hill population of Fd<sub>red</sub> followed by electron transfer to Flv1/3 and O<sub>2</sub> reduction could be significant so as to lead to a NADPH consumption faster in WT than in  $\Delta flv1/3$ . This point was checked by numerical simulations and it was found that the NADPH decay should be only weakly accelerated by the presence of Flv1/3 (Fig. SI6/B). However, in all our measurements, such a difference was not observed as the NADPH decay kinetics were either quasi-identical in both strains (Fig. 1 and SI5) or were a little (10% at most) faster in  $\Delta flv1/3$ . This must be attributed to the fact that a non-identified NADPH consuming process is slightly faster in  $\Delta flv1/3$  than in WT (Fig. SI6/D). The presence of a lag in NADPH decay kinetics, which is correlated to the presence of FeS<sub>red</sub>, has also been reproduced in our simulations (Fig. SI6).

### Rates of Fd<sub>red</sub>/FeS<sub>red</sub> reoxidation by different partners including FNR, Flv1/3 and NDH-1L.

From the comparison of FeS<sub>red</sub> decay in WT and  $\Delta Flv1/3$  (monoexponential rates of 13.2 and 2.3 s<sup>-1</sup>, respectively), one can deduce a rate  $k_{Flv}$  of 11 s<sup>-1</sup> for FeS<sub>red</sub> oxidation by Flv1/3 under the conditions of Fig. 4. As discussed above, the largest part of the light-induced FeS<sub>red</sub> signal of Fig. 4 probably arises from the terminal PSI acceptors F<sub>A</sub> and F<sub>B</sub> (Table 2). Therefore  $k_{Flv}$  corresponds either to (F<sub>A</sub> F<sub>B</sub>)<sub>red</sub> oxidation or to Fd<sub>red</sub> oxidation by Flv1/3 which would limit the rate of (F<sub>A</sub> F<sub>B</sub>)<sub>red</sub> oxidation:

1) In the former case, Fd reduction by (F<sub>A</sub> F<sub>B</sub>)<sub>red</sub>, a process occurring in the  $\mu$ s-ms time range, would outcompete Flv1/3 reduction by (F<sub>A</sub> F<sub>B</sub>)<sub>red</sub> by several orders of magnitude. This last process would thus be significant only when Fd is completely reduced;

2) In the latter case, FNR would be the main competitor of Flv1/3 for Fd<sub>red</sub> oxidation. The *in vivo* kinetics of FNR reduction by Fd<sub>red</sub> are not known precisely but the kinetics of NADPH formation following a single saturating turnover flash have been measured in *Syn. 6803* cells. Half of the maximum flash-induced NADPH signal is formed at 8 ms [39], which indicates that the rate  $k_{FNR}$  of single electron FNR reduction by Fd<sub>red</sub> should be significantly faster than 100 s<sup>-1</sup>. Spectroscopic evidence for a high rate of FNR reduction can also deduced from

the NIR FeS<sub>red</sub> signal which exhibits a sigmoidal shape when the NADP pool is mostly oxidized (Fig. 6). This underlines that the efficiency of Flv1/3 reduction by Fd<sub>red</sub> should increase considerably when both FNR and the NADP pool are fully reduced.

We also detected no significant difference in kinetics of FeS<sub>red</sub> decay between WT and  $\Delta NdhD1/D2$ , in which the NDH-1L complex is absent (Fig. 7B). This result could have been predicted just from the comparison of WT and  $\Delta flv1/3$ , as Flv1/3 is the dominant partner for oxidation of FeS<sub>red</sub> under both conditions of not active CBC and fully reduced NADP pool. Under these conditions, the oxidation rate of FeS<sub>red</sub> by all other partners is about 2 s<sup>-1</sup>. Even if NDH-1L contributes for a significant part to this rate, it would make little difference between WT and  $\Delta NdhD1/D2$  and such a small difference may have escaped detection in our measurements. A more precise comparison of FeS<sub>red</sub> oxidation using extensive averaging may be required to decide this. It has been previously found that the absence of NDH-1L impedes growth under high-light conditions [53-56]. Moreover, a slow *in vivo* rate of Fd<sub>red</sub> oxidation by NDH-1L appears to contradict the high rate of NDH-1L reduction by Fd<sub>red</sub> that was measured *in vitro* [27]. Indeed the second-order rate constant of single NDH-1L reduction was found to be larger than that of FNR reduction. Several non-exclusive possibilities, which could also be related to the different strains involved (*Syn. 6803 vs T. elong.*), may explain these apparent discrepancies: 1) firstly, FNR is in large excess over NDH-1L; 2) membrane reorganization favoring recognition efficiency between Fd<sub>red</sub> and NDH-1L (*e.g.* under high light) increases NDH-1L reduction by Fd<sub>red</sub>, whereas NDH-1L is distant from PSI under our conditions of measurement; 3) Fd<sub>red</sub> oxidation by NDH-1L concerns a minor part of Fd having escaped specific identification during our measurements, *e.g.* by involving a small percentage of PSI associated to NDH-1L in a supercomplex [29].

### Electron transfer scheme and conclusions

Our work shows that Fd<sub>red</sub> is the electron donor to Flv1/3 for reducing water to oxygen. However, we cannot exclude that (F<sub>A</sub> F<sub>B</sub>)<sub>red</sub>, the reduced PSI terminal acceptor, can directly reduce Flv1/3. We also obtained evidence that (F<sub>A</sub> F<sub>B</sub>) can be highly reduced under continuous illumination and that recombination between P700<sup>+</sup> and F<sub>Xred</sub> is observable *in vivo*. Future work with KLAS-NIR may be useful to clarify the correlation between the redox state of Fd/(F<sub>A</sub> F<sub>B</sub>) and PSI acceptor side limitation [57], under different conditions including those where the CBC is activated.

Fig. 8 shows an electron transfer scheme involving the acceptor side of PSI. Part A shows the electron transfer rates which were deduced from the present study, corresponding to experimental conditions where FNR and the NADP pool are fully reduced, *i.e.* just after a short illumination with a poorly activated or inactivated CBC (Figures 1, 3-4). From the kinetics of slow FeS<sub>red</sub> oxidation in  $\Delta flv1/3$ , it can be deduced that Fd<sub>red</sub> is very slowly oxidized by partners other than FNR (and Flv1/3 in WT):  $k_p + k_{inact.} \approx 2 \text{ s}^{-1}$ , where  $k_{inact.}$  is the oxidation rate of NADPH which, *via* FNR, leads to Fd<sub>red</sub> oxidation (due to residual CBC activity and any processes consuming directly NADPH). In turn, it means that  $k_p$  is less than 2 s<sup>-1</sup>.  $k_p$  incorporates all processes of Fd<sub>red</sub> oxidation by different partners, mostly NDH-1L and soluble partners such as ferredoxin-thioredoxin reductase and redox enzymes for sulfur and nitrogen assimilation, among which nitrite reductase (NiR). One essential issue is to quantify  $k_p$  under different types of experimental conditions, as  $k_p$  may vary considerably according to these conditions. As an example, we discuss here the specific case of NiR: it may occur that in the conditions of Figures 3-4, both NiR and its substrate nitrite are fully reduced at the end of the illumination period, as it is the case for FNR and its substrate NADP (Fig. 1). If true, this may lead to a very slow Fd<sub>red</sub> oxidation by NiR (rate  $k_{pNiR}$ ) which would be limited by ammonium (the product of NiR activity) consumption. Conversely, the  $k_{pNiR}$  rate may be much larger in other circumstances, *e.g.* either transiently at the onset of illumination (before full reduction of nitrite) or under a stationary state of illumination, where it is also possible that it is upward regulated. The possibility that  $k_p$  is much larger than 2 s<sup>-1</sup>, *e.g.* when the CBC is activated and the NADP pool partially oxidized, is illustrated in part B of Fig. 8.

With regard to the above discussion, the question should be asked whether NDH-1L reduction by Fd<sub>red</sub> (rate  $k_{pNDH1L}$ ) could also be indirectly limited by later steps of the catalytic cycle under the conditions of Figures 3-4, *e.g.* by the availability of oxidized plastoquinone, its final electron acceptor. If there is no such limitation, it would mean that  $k_{pNDH1L}$  is mostly limited by electron transfer from Fd<sub>red</sub> to NDH-1L. With  $k_{pNDH1L} < k_p = 2 \text{ s}^{-1}$ , this would question the efficiency of NDH-1L involvement in a significant electron flux under light, and more

generally the efficiency of cyclic electron flow in *Syn. 6803*, at least upon onset of actinic light. Future studies aimed at determining the precise  $\text{FeS}_{\text{red}}$  kinetics in different mutants and at various physiological conditions should contribute to answer this question.

### **Acknowledgements**

This work was supported by JST CREST Grant Number JPMJCR1503 (Japan), by the LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS) and the French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INSB-05. It is also partially supported by a grant from the Agence Nationale de la Recherche (RECYFUEL project ANR-16-CE05-0026). G.S. is supported by a JSPS overseas research fellowship (201860126).

Table 1 : Relative differential absorption coefficients in *Syn. 6803*

	Coefficients at 902.5 nm
P700 <sup>+</sup>	1
Pcy <sub>ox</sub> – Pcy <sub>red</sub>	0.252 <sup>a</sup> ± 0.005 <sup>c</sup>
(F <sub>A</sub> F <sub>B</sub> ) <sub>1red</sub> - (F <sub>A</sub> F <sub>B</sub> ) <sub>ox</sub>	-0.044 <sup>b</sup> ± 0.010
Fd <sub>red</sub> - Fd <sub>ox</sub>	-0.026 <sup>b</sup> ± 0.006

Coefficients at 902.5 nm (840 – 965 nm) were calculated from previous in vitro measurements [41] and are given by reference to a value of 1 for P700<sup>+</sup>.

<sup>a</sup>Derived from the tobacco coefficient by multiplication by a factor of 1.218 which corresponds to the ratio of the P700<sup>+</sup> absorption coefficients of tobacco and *Syn. 6803* (4.75 and 3.9 M<sup>-1</sup>cm<sup>-1</sup>, respectively; Table 1 in [41])

<sup>b</sup>Derived from the *T. elong.* coefficient by multiplication by a factor of 1.218 which corresponds to the ratio of the P700<sup>+</sup> absorption coefficients of *T. elong.* and *Syn. 6803* (4.75 and 3.9 M<sup>-1</sup>cm<sup>-1</sup>, respectively)

<sup>c</sup>Uncertainties values were taken from [41].

Table 2: Maximum signals and relative amounts of the different electron-transfer components in WT and  $\Delta flv1/3$  cells.

	WT		$\Delta flv1/3$	
P700 max. value	1.26		1.37	
Calculated signal <sup>a</sup>	1.16		1.19	
Amplification factor <sup>b</sup>	1.09		1.15	
Pcy max. value	0.73		0.77	
$n_{Pcy}$ <sup>c</sup>	2.30 ± 0.05		2.23 ± 0.05	
FeS <sup>d</sup> max. value	-0.15	(100%)	-0.17	(100%)
(F <sub>A</sub> F <sub>B</sub> ) contribution <sup>e</sup>	-0.111 ± 0.025	(74%)	-0.121 ± 0.027	(71%)
Fd contribution <sup>f,h</sup>	-0.039	(26%)	-0.049	(29%)
$n_{Fd}$ <sup>g,h</sup>	1.1		1.4	

Maximum values were determined from Fig. 2 and are given in ( $\Delta I/I \times 10^{-3}$ ) at 902.5 nm. Cells concentrations were 12.5 and 12.8  $\mu\text{g Chl./mL}$  for WT and  $\Delta flv1/3$ , respectively. Relative stoichiometries ( $n_{Pcy}$  and  $n_{Fd}$ ) are given by reference to that of P700<sup>+</sup> (= Pcy/PSI and Fd/PSI ratios).

<sup>a</sup>The calculated P700 signal is obtained by assuming a chl./P700 ratio of 108 and an absorption coefficient of 3,900  $\text{M}^{-1}\text{cm}^{-1}$ .

<sup>b</sup>Amplification factor = P700 max. value/Calculated signal.

<sup>c</sup>The number of Pcy per PSI (P700) is calculated using a relative coefficient of  $0.252 \pm 0.005$  (Table 1): e.g. for WT,  $n_{Pcy} = (0.73/1.26)/0.252$

<sup>d</sup>FeS identifies both Fd and the PSI iron-sulfur centers F<sub>A</sub> and F<sub>B</sub>

<sup>e</sup>The (F<sub>A</sub> F<sub>B</sub>) contribution is calculated for reduction of both clusters using a relative coefficient (single reduction) of  $-0.044 \pm 0.010$  (Table 1) : e.g. for WT, (F<sub>A</sub> F<sub>B</sub>) contribution =  $-0.044 \times 1.26 \times 2$ .

<sup>f</sup>Fd contribution = FeS max. value - (F<sub>A</sub> F<sub>B</sub>) contribution

<sup>g</sup>Using a relative coefficient of -0.026 (Table 1), which gives, for 1 Fd per PSI, signals of -0.033 and -0.036 for WT and  $\Delta Flv1/3$ , respectively.

<sup>h</sup>Very large relative uncertainties are not given

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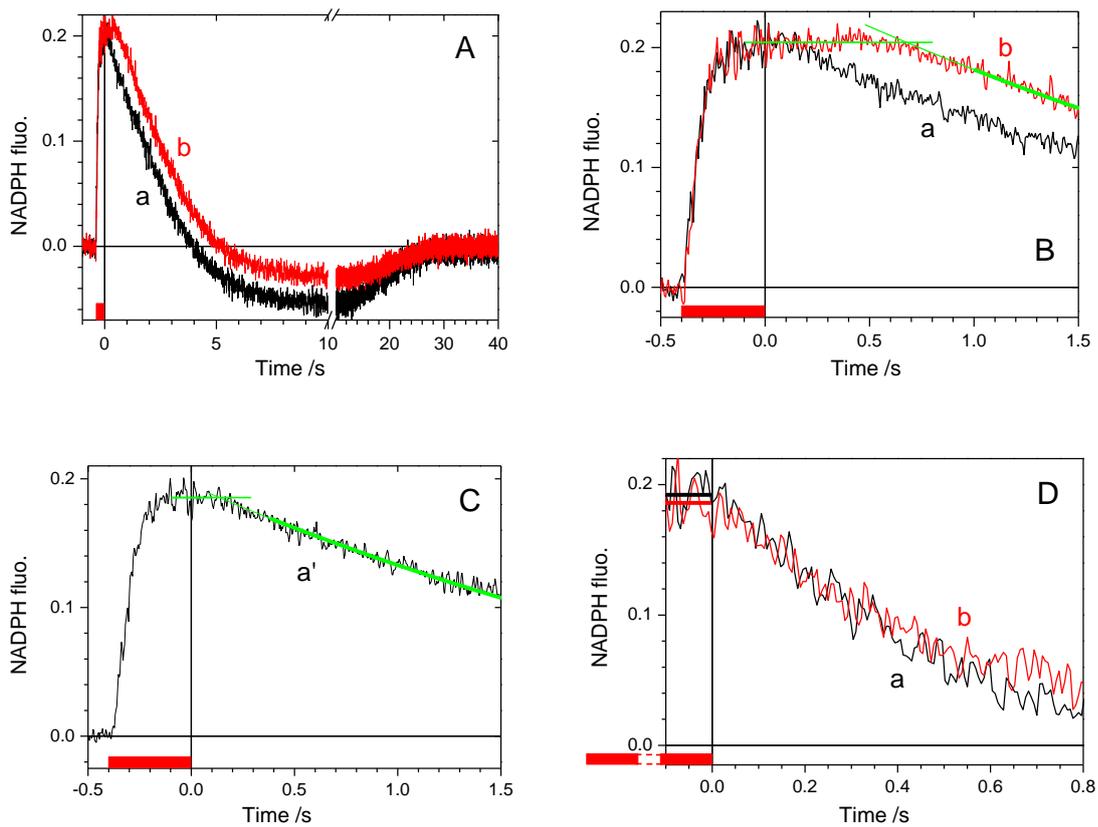


Fig. 1. NADPH fluorescence kinetics in WT and  $\Delta flv1/3$  cells.

Cells were studied at a concentration of 4  $\mu\text{g}$  Chl./ml. The dark fluorescence levels were set at 0 and time was set at 0 at the end of illumination. Illumination time is indicated by a red bar at the bottom of all parts. **A** and **B**. The same signals induced by a 400 ms illumination period are shown on 2 different time scales.  $\Delta flv1/3$  signals (red, traces b) were multiplied by a factor of 1.04 for easier comparison of the kinetics with those of WT (black, traces a). In B, the continuous green line corresponds to a monoexponential fit of  $\Delta flv1/3$  signal (thick line) which was extended to shorter times (thin line). This line crosses the fluorescence level at the end of illumination (horizontal green line) at c. 700 ms after the end of illumination. Actinic light intensity of 700  $\mu\text{moles photons m}^{-2}\text{s}^{-1}$ . **C**. A similar experiment was performed with extensive averaging with WT cells. The same procedure as in B was used to get a crossing point at 140 ms. Averages of 25 (A and B) or 80 measurements (C) separated by 1 min. time intervals. **D**. the same cells as in A and B were later on illuminated for 120 s at an intensity of 350  $\mu\text{moles photons m}^{-2}\text{s}^{-1}$ . The signal levels at the end of illumination (fitted with a constant amplitude between -5 s and 0) are shown as horizontal lines. Averages of 5 measurements separated by 6 min. time intervals. Cells were initially dark-adapted for 8 min.

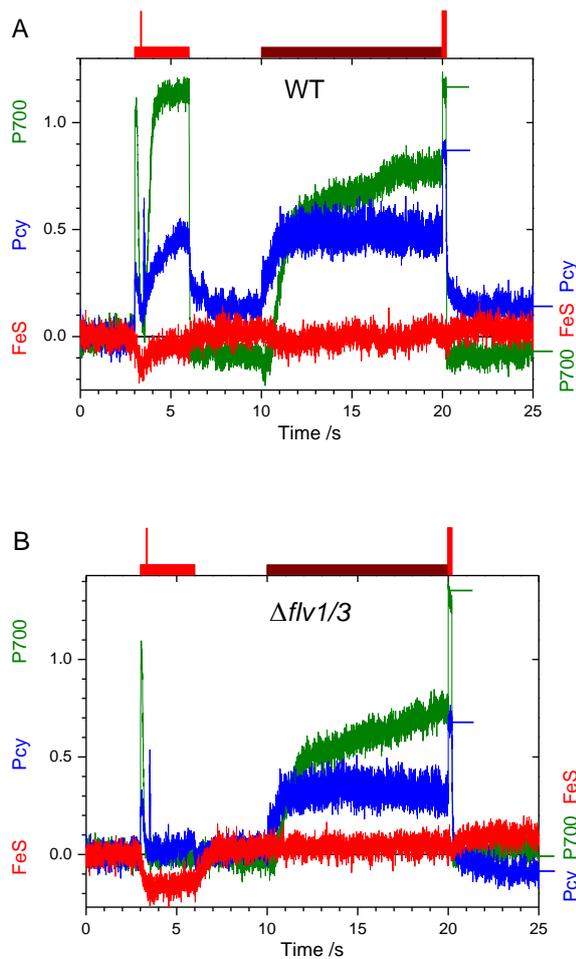


Fig. 2. Maximum amplitudes of P700, Pcy and iron-sulfur signals in WT and  $\Delta flv1/3$  cells determined by NIR absorption.

Signals (P700, green; Pcy, blue; FeS, red) were measured in WT (part A, 12.5  $\mu\text{g Chl./ml}$ ) and  $\Delta flv1/3$  (part B, 12.8  $\mu\text{g Chl./ml}$ ) cells under red actinic light (from 3 to 6 s, 930  $\mu\text{moles photons m}^{-2}\text{s}^{-1}$ ) and just after far red light (from 10 to 20 s) by using MT pulses (25,000  $\mu\text{moles photons m}^{-2}\text{s}^{-1}$ ) of 30 ms duration (under red light, MT begins at 3.35 s) or 200 ms duration (MT begins when far red light is switched off). Horizontal marks indicate the maximum intensity levels under light or the zero levels at the end of illumination (P700 and Pcy). The calculated signals are given in Table 2. Cells were initially dark-adapted for 8 min. One single measurement in each case. Vertical scales are in  $(\Delta I/I \times 10^{-3})$  at 902.5 nm. The illumination protocol is indicated at the top of the figures (red and dark red colors for red and far red light, respectively).

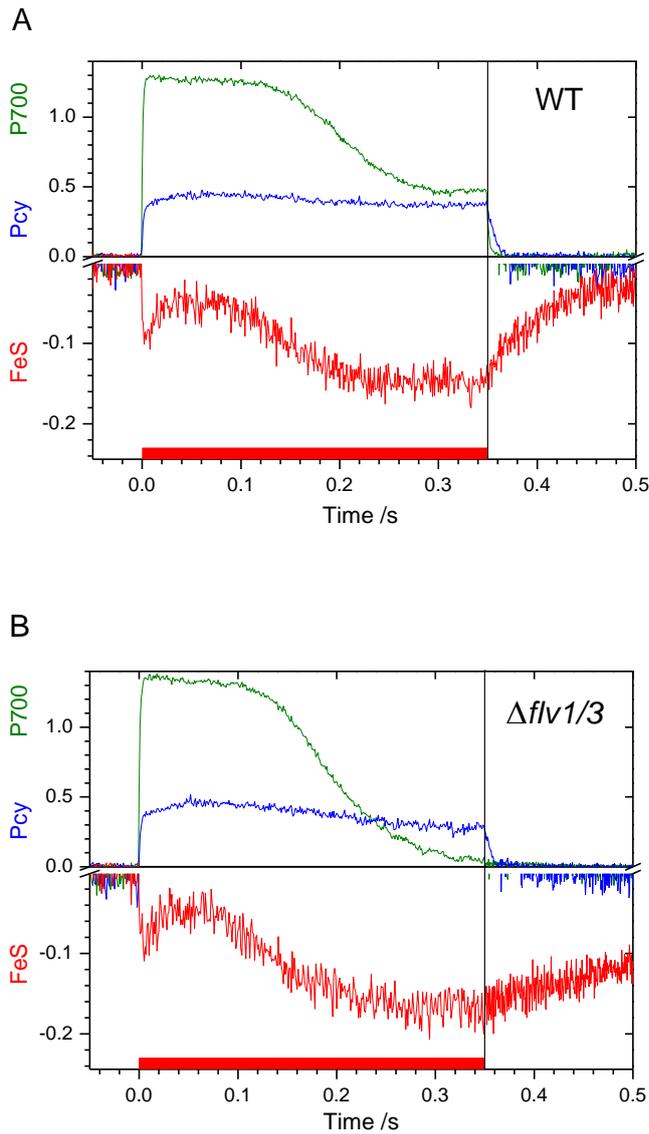


Fig. 3. P700, Pcy and FeS signals in WT and  $\Delta flv1/3$  cells under and after a short illumination.

The same cell suspensions used in Fig. 2 were submitted to high intensity red illumination ( $8,100 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ ) for 350 ms, a time at which a minimum is observed in the WT P700<sup>+</sup> signal. Note that the Y-scales are different for the upper and lower parts of each figure. Averages of 20 measurements, separated by 1 min. time intervals. Cells were initially dark-adapted for 8 min. The red rectangles at the bottom of the kinetics indicate the periods of illumination. Vertical scales are given in  $(\Delta I/I \times 10^{-3})$  at 902.5 nm.

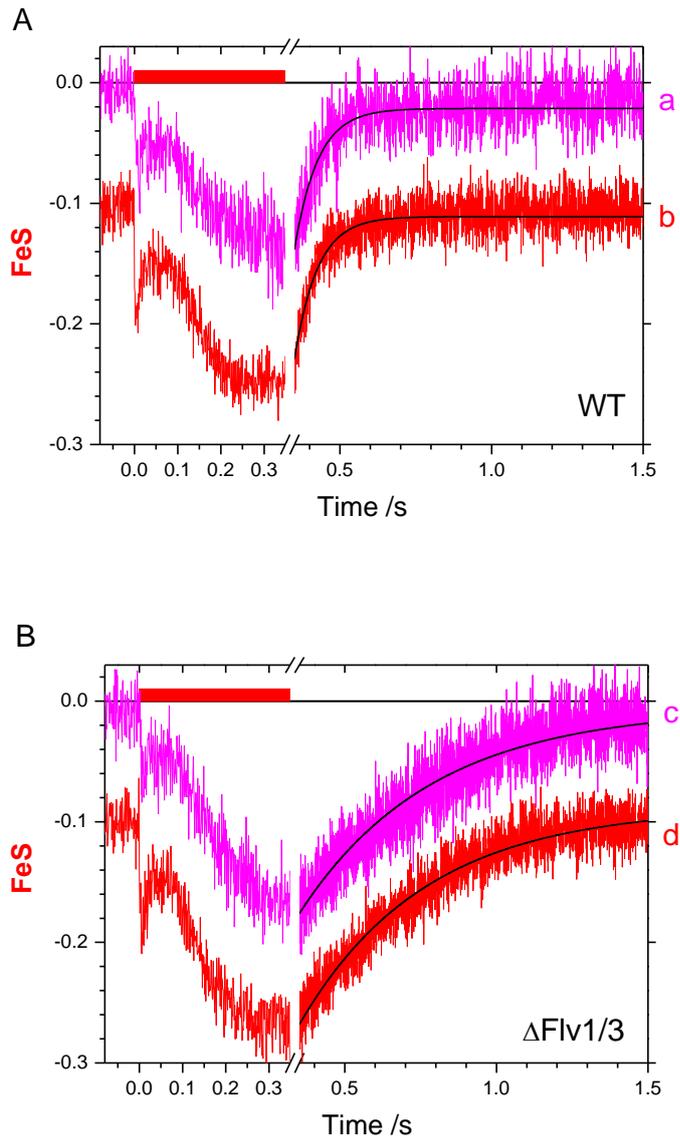


Fig. 4. FeS signals in WT and  $\Delta flv1/3$  cells under and after a short illumination.

Red traces are those of Fig 3 (shifted by -0.1) and magenta ones were obtained with the same samples under similar conditions but at lower light intensity ( $1,170 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ ). Note a break in the X-scale. All decay kinetics were fitted (black traces) with single exponentials ( $A \times \exp[-k(t-0.35)] + B$ ), resulting in rates of  $13.1$ ,  $13.3$ ,  $2.3$  and  $2.4 \text{ s}^{-1}$  for traces a, b, c and d, respectively. The red rectangles above the baseline indicate the periods of illumination. Cells were initially dark-adapted for 8 min. Vertical scales are given in  $(\Delta I/I \times 10^{-3})$  at  $902.5 \text{ nm}$ .

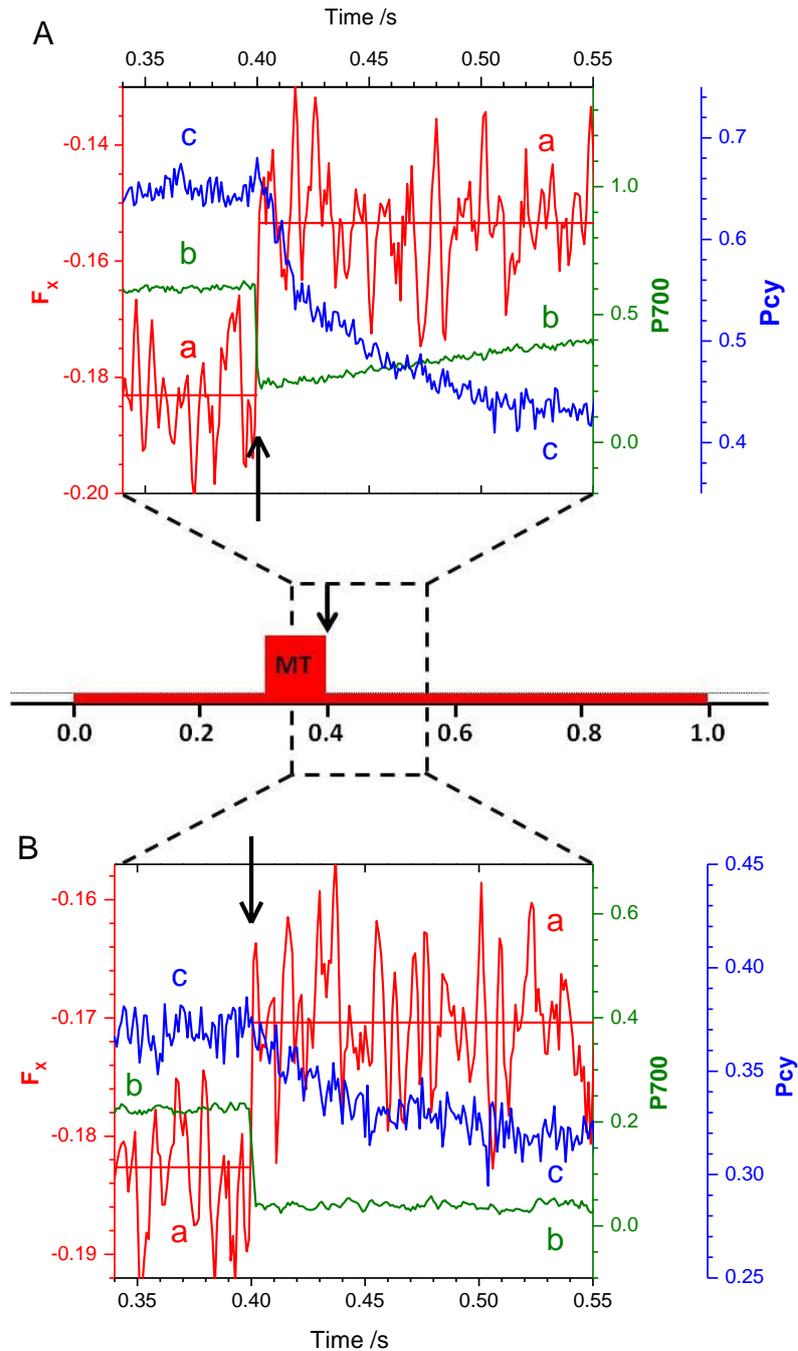


Fig. 5. Evidence for recombination between  $P700^+$  and  $F_{xred}$

WT (A) and  $\Delta flv1/3$  (B) cells at concentrations identical to those of Figures 3 and 4 were submitted from 0 to 1 s to red actinic light ( $2,200 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ ) and between 0.3 and 0.4 s to a 100 ms MT pulse ( $40,000 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ ). Kinetics of P700 (trace b, green), Pcy (trace c, blue) and FeS/ $F_x$  (trace a, red) are shown at the end of the MT pulse, which is indicated by a vertical arrow. The middle part of the figure shows the illumination sequence with the dashed rectangle showing the region which is zoomed in the upper and lower part. The Y-scales of the 3 species are shown with different colors in  $(\Delta I/I \times 10^{-3})$  at 902.5 nm with all signals set to 0 before illumination. For part B, the Y-scales are expanded twice relative to part A. In each part, the full vertical scales of the different species (P700, Pcy and  $F_x$ ) are proportional to their relative absorption coefficients (Table 1: 1, 0.252 and -0.044 for P700, Pcy and  $(F_A F_B)$ , respectively), so that the signal amplitudes can be directly compared. Thus recombination between  $P700^+$  and  $F_{xred}$  should give similar apparent

amplitudes, if  $F_x$  reduction gives a signal identical to that of single ( $F_A F_B$ ) reduction. Cells were initially dark-adapted for 8 min. Averages of 40 measurements, separated by 1 min. time intervals.

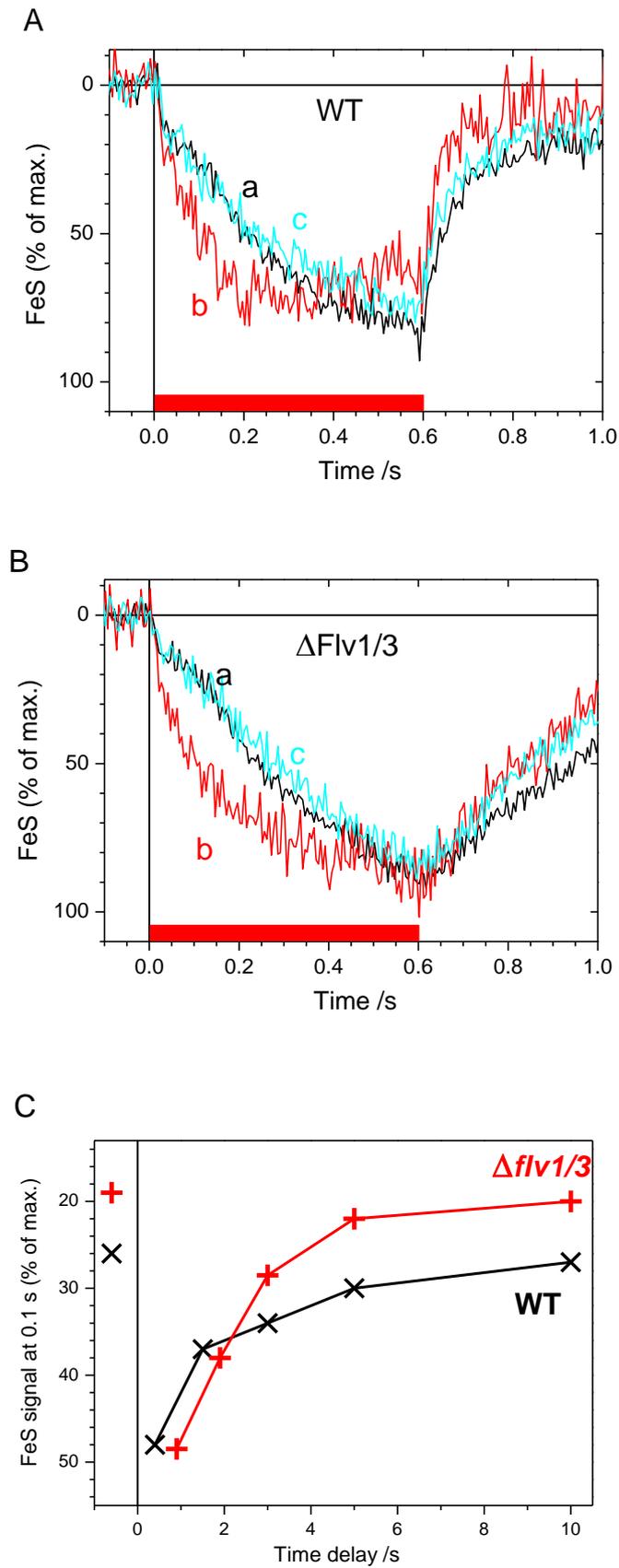


Fig. 6. Dependence of the kinetics of FeS reduction on the NADPH level.

FeS reduction was measured during two consecutive periods of 0.6 s red light illumination (hv) periods at an intensity of  $250 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ . **A.** WT, a (black), first hv; b (red), second hv starting 0.4 s after the end of the 1<sup>st</sup> hv; c (cyan), average of signals with the second hv starting 5 and 10 s after the end of the 1<sup>st</sup> hv. **B.**  $\Delta flv1/3$ , similar signals as in A, except that trace b was measured with a delay of 0.9 s, which is necessary for the almost complete reoxidation of FeS. **C.** The FeS signal, which was measured at 0.1 s after the beginning of the 2<sup>nd</sup> hv, is plotted as a function of the delay time between the end of the 1<sup>st</sup> hv and the beginning of the 2<sup>nd</sup> hv. The 1<sup>st</sup> hv signal at 0.1 s is shown on the upper left of the figure ( $t < 0$ ). WT (black crosses, x) and  $\Delta flv1/3$  (red plus signs, +). Both strains were at *c.* 9  $\mu\text{g chl./ml}$ . Signals are plotted as a function of the maximum FeS signal intensities that were recorded as in Fig. 2. Traces a, b and c are averages of 100, 20 and 40 measurements, respectively. Measurements are separated by 1 min. time intervals. The red rectangles at the bottom of the kinetics (A/B) indicate the periods of illumination. Cells were initially dark-adapted for 8 min. Vertical scales in  $(\Delta I/I \times 10^3)$  at 902.5 nm.

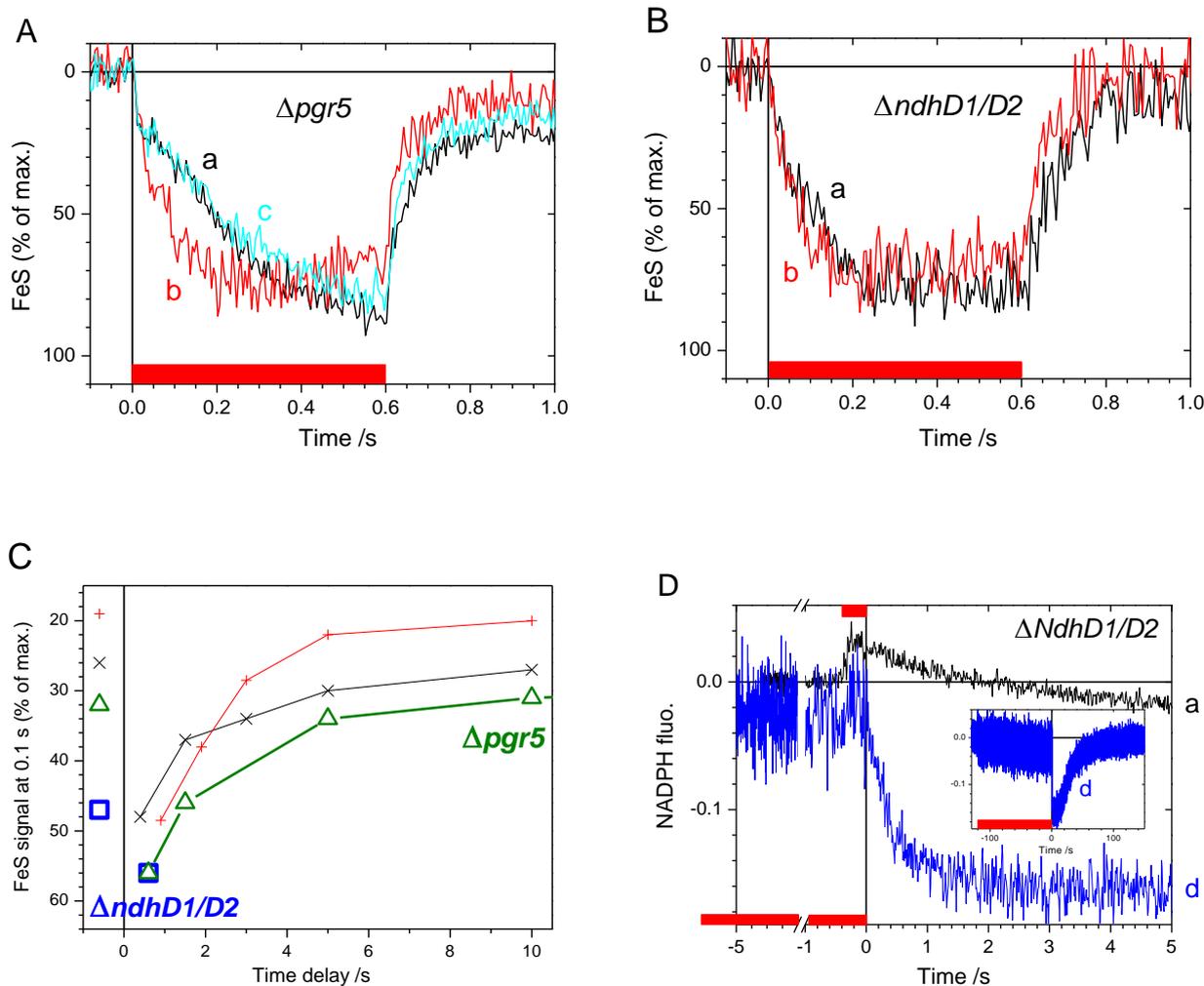
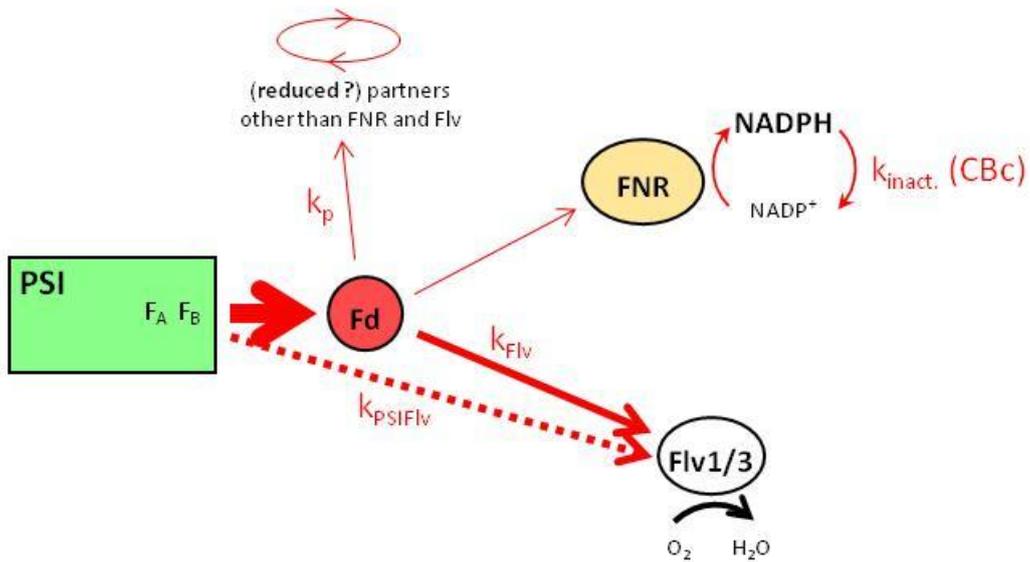


Fig. 7. Kinetics properties of  $\Delta pgr5$  and  $\Delta ndhD1/D2$  cells.

Parts **A** and **B**: Experimental conditions are identical to those of Fig. 6. FeS reduction was measured during two consecutive periods of 0.6 s red light illumination ( $h\nu$ ) periods at an intensity of  $250 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ . Trace a: 1<sup>st</sup>  $h\nu$ . Trace b: 2<sup>nd</sup>  $h\nu$  beginning 0.6 s after end of 1<sup>st</sup>. Trace c (for  $\Delta pgr5$ ): 2<sup>nd</sup>  $h\nu$  beginning 5, 10 and 20 s (signal average) after end of 1<sup>st</sup>  $h\nu$ .  $\Delta pgr5$ : averages of 90, 20 and 50 measurements for traces a, b and c, respectively.  $\Delta ndhD1/D2$ : averages of 10 measurements for both traces. Individual measurements are separated by 1 min. time intervals. The red rectangles indicate the period of illumination. Cells were initially dark-adapted for 8 min. Signals are shown in percent of the maximum FeS<sub>red</sub> signal amplitude. **C**. The FeS signal, which was measured at 0.1 s after the beginning of the 2<sup>nd</sup>  $h\nu$ , is plotted as a function of the delay time between the end of the 1<sup>st</sup>  $h\nu$  and the beginning of the 2<sup>nd</sup>  $h\nu$ . The 1<sup>st</sup>  $h\nu$  signal at 0.1 s is shown on the upper left of the figure ( $t < 0$ ).  $\Delta pgr5$  (green triangles) and  $\Delta ndhD1/D2$  (blue squares). Data of Fig. 6C are also shown with thin lines and small symbols for the sake of comparison (black x for WT and red + for  $\Delta flv1/3$ ). **D**. NADPH fluorescence kinetics in  $\Delta ndhD1/D2$  cells. Trace a (black): red actinic light illumination of 400 ms was given to a dark-adapted sample ( $700 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ , average of 30 measurements separated by 1 min. time intervals). Trace d (blue): the sample was later on submitted to a prolonged red light illumination of 120 s ( $350 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ , average of 5 measurements separated by 6 mins. time intervals). For both traces, the fluorescence levels in the dark before illumination were set at 0 and time 0 corresponds to the end of illumination. The inset shows the fluorescence kinetics corresponding to trace d on a longer time scale. The red rectangles at the bottom of the kinetics (and at the top for trace a) indicate the periods of illumination. Chl. concentration (D) was c.  $4 \mu\text{g/ml}$ .



**A** NADP pool and FNR fully reduced, dark just after short light



**B** NADP pool partially oxidized

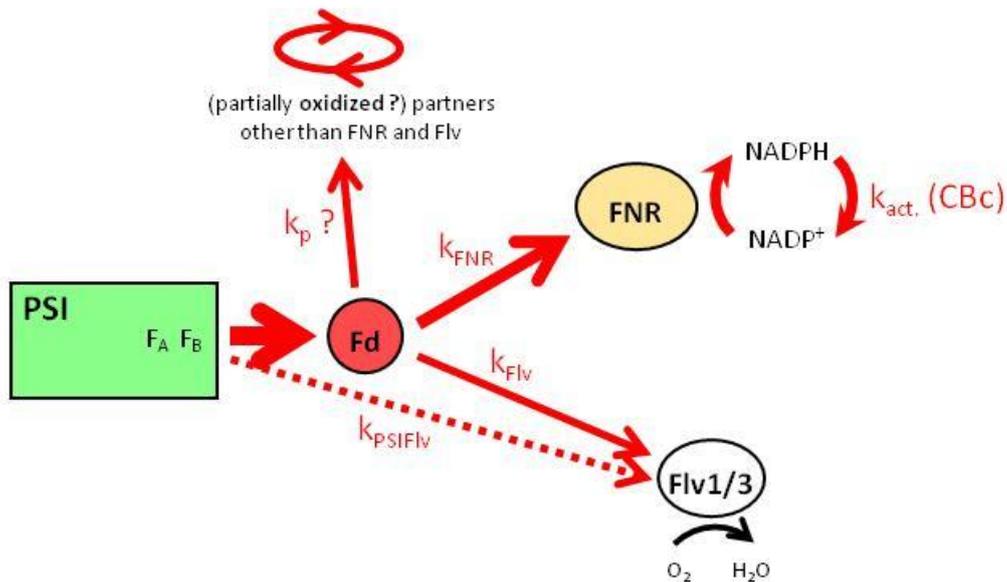


Fig. 8. Electron transfer scheme involving the acceptor side of PSI.

Part **A** corresponds to the situation encountered in Figures (1, 3-4), where FNR and the NADP pool are fully reduced and with a poorly activated or inactivated CBc.  $Fd_{red}$  has many partners other than FNR or Flv1/3 (NDH1-L, enzymes involved in nitrogen assimilation, etc...) giving a global rate  $k_p$  of  $Fd_{red}$  oxidation. From the present KLAS-NIR measurements:  $k_{Flv} = 11 \text{ s}^{-1}$ ;  $k_p + k_{inact.} = 2.3 \text{ s}^{-1}$ .  $k_{inact.}$  is defined as a  $Fd_{red}$  oxidation rate corresponding to the slow rate of NADPH oxidation which limits the

electron transfer pathway:  $Fd \rightarrow FNR \rightarrow NADPH \rightarrow \text{substrates}$ . The relative values of  $k_p$  and  $k_{inact}$  cannot be deduced from the present work. The dotted arrow from  $F_B$  to Flv indicates that direct reduction of Flv by PSI cannot be excluded from our data. Part **B** corresponds to situations where the NADP pool is not completely reduced because of Cbc activation (rate  $k_{act}$ .) and where the rate  $k_p$  is hypothetically assumed to be larger than in A (see text). From the kinetics of NADPH photoreduction:  $k_{FNR} \geq 100 \text{ s}^{-1}$ .

## Supplementary Figures

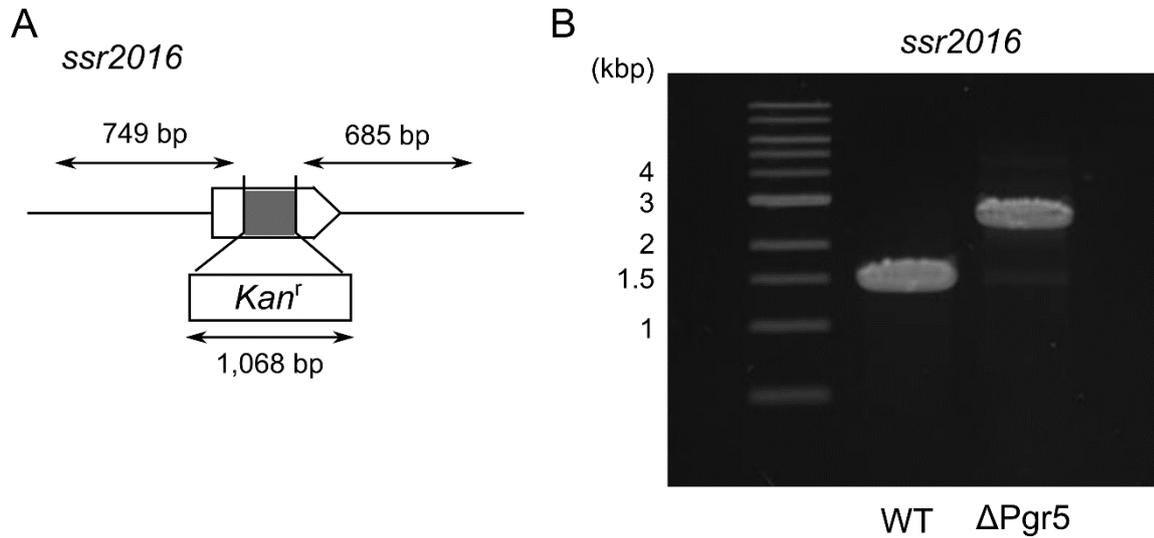


Fig. S11. Insertional inactivation of the *pgr5* gene in *Syn. 6803*.

(A) Physical map of the insertion site of antibiotic resistance cassette in  $\Delta$ *pgr5*. The arrowheads indicate the locations of PCR primers. The lengths of the amplified DNA fragments are shown. The full length of the gene is 198 bp. The shaded box indicates the removed genome DNA region (33 bp). (B) DNA fragments amplified by PCR showing complete segregation of the inactivated gene.

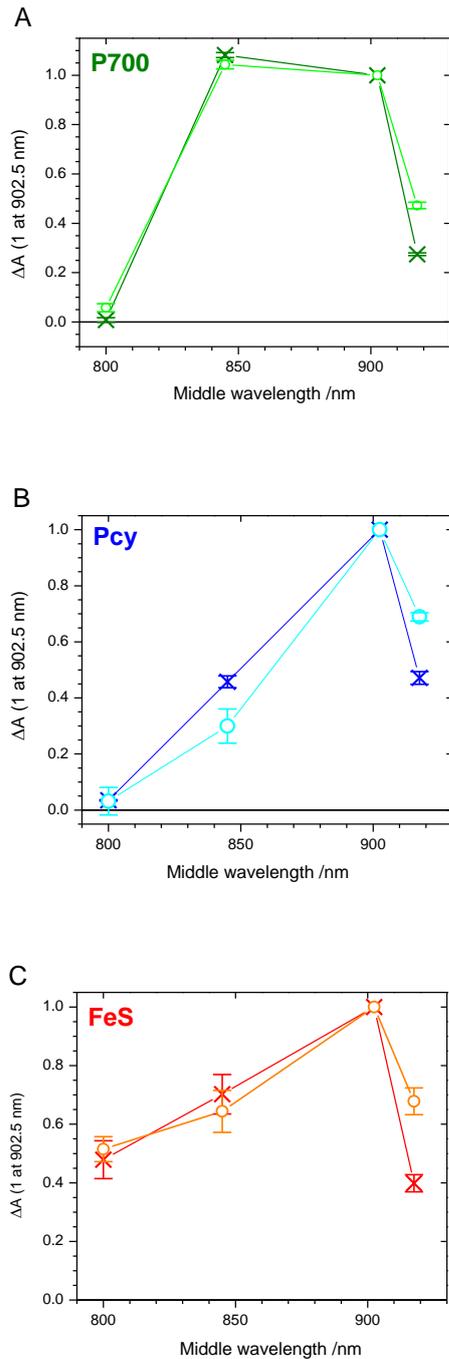


Fig. S12. Differential model plots of P700 (A), Pcy (B) and FeS (C).

All spectra are normalized to 1 at the middle wavelength of 902.5 nm. The spectra shown as crosses were used in the present study and were measured in Kobe university with the *Syn. 6803* WT and  $\Delta flv1/3$  strains from Kobe (averages of 3 measurements). The spectra shown as open circles were measured in the Saclay laboratory with the same WT strain (averages of 2 measurements). All measurements were performed at 25°C.

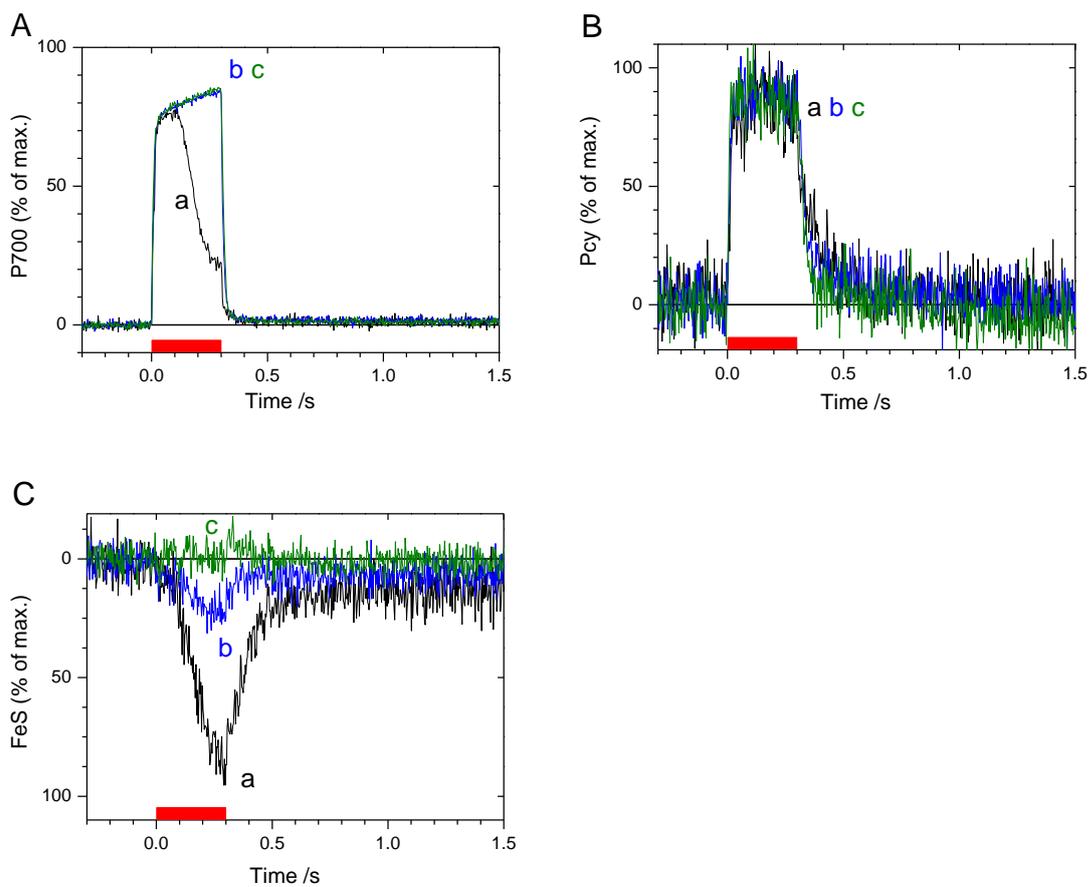


Fig. S13. Effect of methyl viologen (MV) on P700 (A), Pcy (B) and FeS (C) signals.

300 ms pulses of high intensity ( $14,500 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ ) were given to a concentrated suspension of WT cells (*c.*  $45 \mu\text{g chl. ml}$ ). Traces a were recorded before MV addition. Then 3 measurements were averaged between 7 and 9 min. (traces b) and between 18 and 20 min. (traces c) after addition of  $300 \mu\text{M}$  MV. Measurements are separated by 1 min. time intervals.

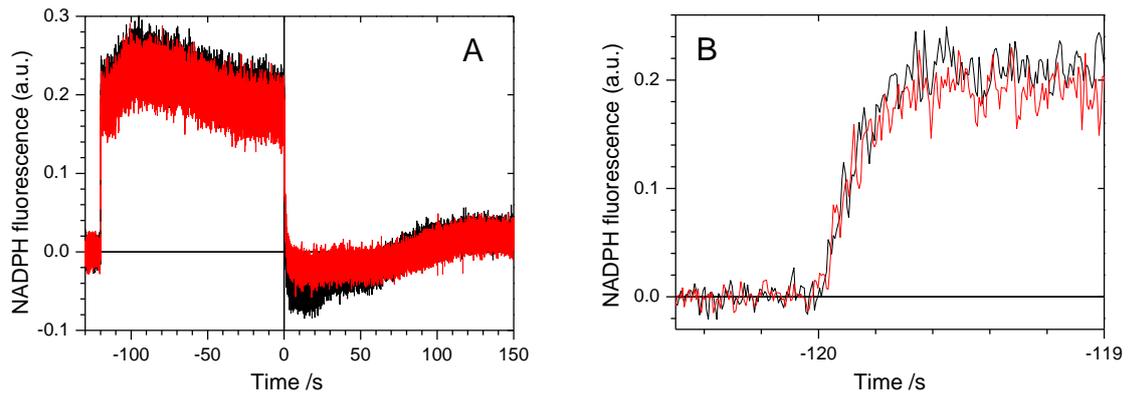


Fig. S14. NADPH fluorescence kinetics with a 120 s illumination period.

The same cell suspensions of WT (black traces) and  $\Delta fiv1/3$  (red traces) as those of Fig. 1 were later on illuminated for 120 s at a light intensity of  $350 \mu\text{moles photons m}^{-2}\text{s}^{-1}$ . The dark fluorescence levels before illumination were set at 0 and time origin was set at the end of illumination. **B** is a zoomed view of **A** at the onset of illumination. The decay kinetics at the end of illumination are shown in Fig. 1D.

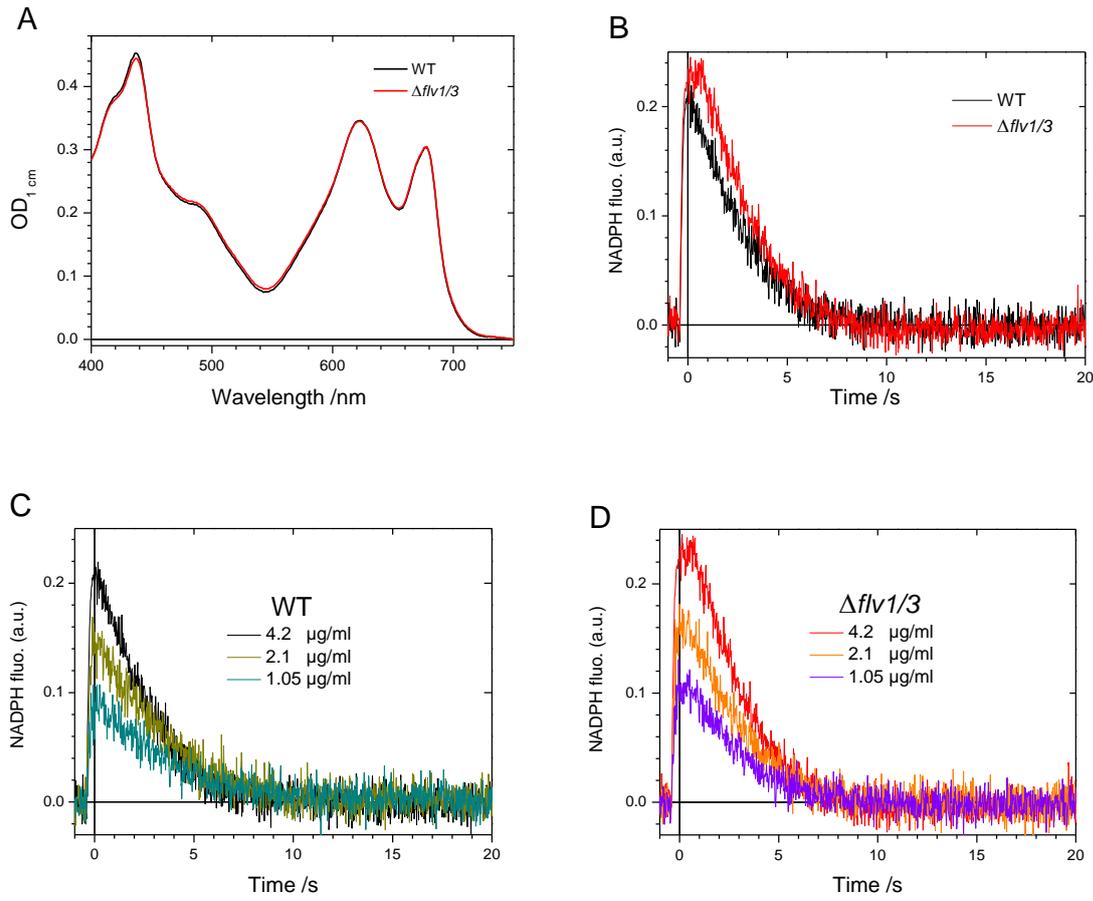


Fig. S15. NADPH fluorescence kinetics at different chlorophyll concentrations. Cell suspensions of WT and  $\Delta flv1/3$  were adjusted at the same chlorophyll concentration of 4.2  $\mu\text{g}/\text{mL}$  by measuring their absorption spectra (A; Shimakawa et al., 2020) before recording light-induced NADPH fluorescence signals (B). Cells were then diluted 2 and 4 times before repeating the measurements (C and D). Signal sizes decrease with chlorophyll content, although less than linearly, as previously reported in this range of chlorophyll concentrations (Kauny and Sétif, 2014). Measurements conditions are similar to those of Fig. 1A, except only 10 measurements were averaged with time intervals of 3 mins. between consecutive measurements. With this time interval, no signal undershoot was observed contrary to Fig. 1A (1 min. time intervals). The  $\Delta flv1/3$  to WT ratio of signals is 1.10.

J. Kauny, P. Sétif (2014) Biochim. Biophys. Acta 1837:792-801.

G. Shimakawa, P. Sétif, A. Krieger-Liszkay (2020) Photosynth. Res. 144:63-72.

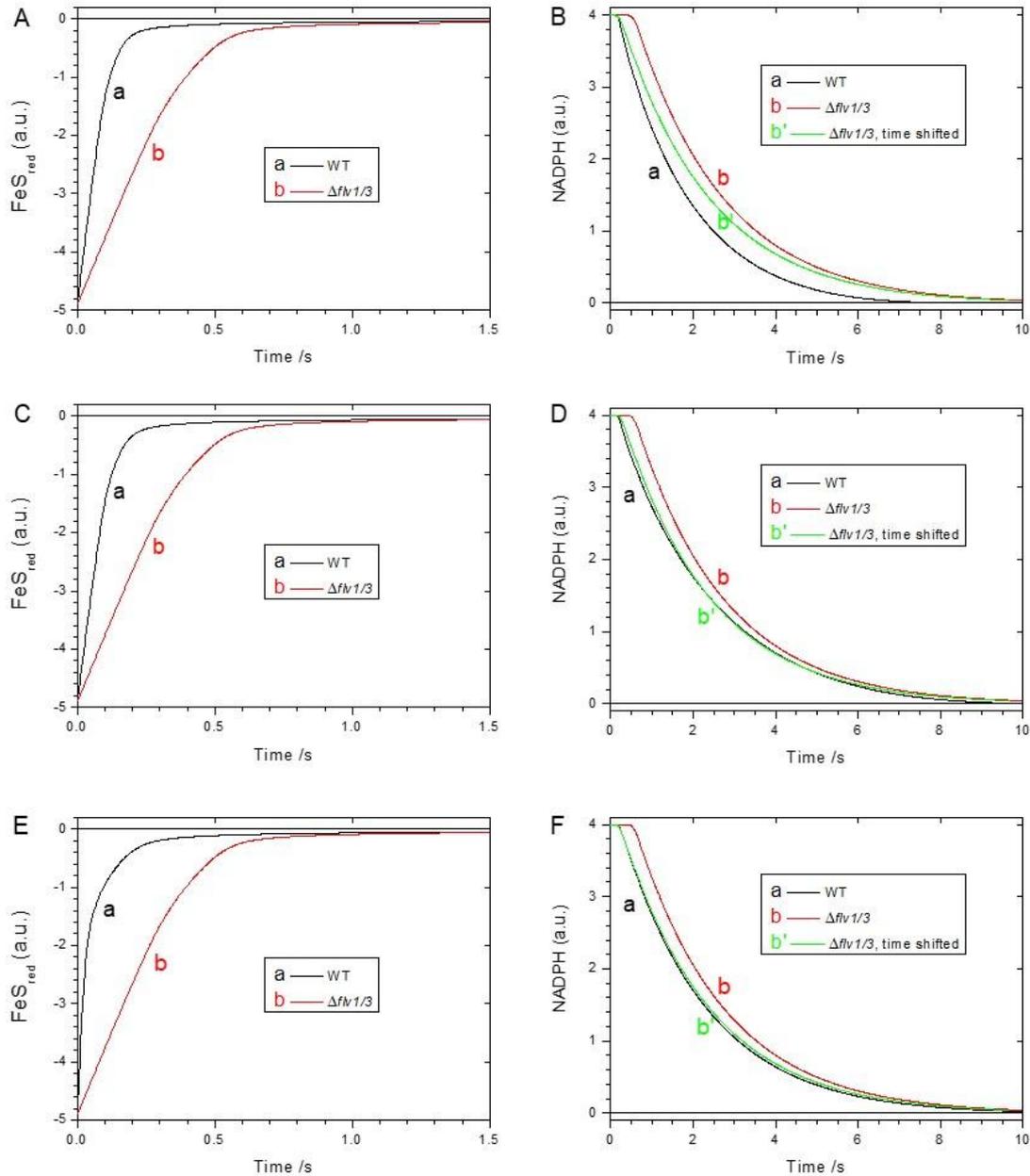


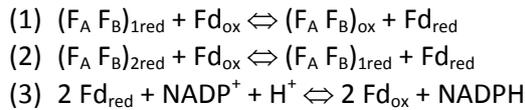
Fig. S16. Simulations of  $\text{FeS}_{\text{red}}$  and NADPH decay kinetics.

These simulations (Mathematica 6.0, Wolfram Research, Inc., Champaign, IL, 2007) were done to qualitatively reproduce salient features of the kinetic differences between WT and  $\Delta\text{flv}1/3$  strains following a short illumination (Figures 1 and 4), but it was not attempted to quantitatively reproduce these kinetics.

$\text{FeS}_{\text{red}}$  (left, A/C/E) and NADPH (right, B/D/F) decay kinetics in darkness following illumination were simulated (arbitrary units) for three different sets of kinetic rate constants (A/B, C/D and E/F). Traces a (black) and b (red) were calculated for WT and  $\Delta\text{flv}1/3$ , respectively. Traces b' ( $\Delta\text{flv}1/3$ ) of NADPH decay (green) are identical to traces b, except for a time shift of -0.34 s. This shift was chosen so that the initial NADPH decay of traces a and b' occur simultaneously.

**Conditions and parameters common to all cases:** **a)** the Fd to PSI ratio is 1.5. **b)** Fd and  $(F_A F_B)$  are 100% reduced at  $t = 0$ . **c)** Double reduction of  $(F_A F_B)$  leads to a signal =  $2 \times 1.7 = 3.4$  times larger than reduction of 1 Fd (Table 1). **d)** The redox equilibria between  $(F_A F_B)$ , Fd and NADPH are very fast vs

oxidizing reactions, *i.e.* these 3 species are at any time in pre-equilibrium conditions. The equilibrium constants  $K_1$ ,  $K_2$  and  $K_3$ , for the 3 following reactions:



are taken to be 30, 100 and 1100, respectively. This corresponds to the following differences of midpoint potentials:

$$E_m((F_A F_B)_{ox}/(F_A F_B)_{1red}) - E_m(Fd_{ox}/Fd_{red}) = -87 \text{ mV}$$

$$E_m((F_A F_B)_{1red}/(F_A F_B)_{2red}) - E_m(Fd_{ox}/Fd_{red}) = -118 \text{ mV}$$

$E_m(Fd_{ox}/Fd_{red}) - E_m(NADP^+/NADPH) = -90 \text{ mV}$ . In this case, the  $NADP^+/NADPH$  equilibrium involves a 2-electrons reaction, hence the large value of  $K_3$ .

**e)** The NADP pool to PSI ratio is 4.0 and NADP is 100% reduced at  $t = 0$ . This pool size corresponds to the upper limit of the NADP photoreducible pool size that was previously determined in *Syn. 6803* (Kauny and Sétif, 2014). **f)** The total rate  $k_p$  of  $Fd_{red}$  oxidation by partners other than FNR and Flv has a value of  $2 \text{ s}^{-1}$  (see Fig. 8)

**Parameters specific to different cases** (see Fig. 8 for the definition of rate constants):

**Parts A and B:** Flv is reduced by Fd.  $k_{Flv} = 11 \text{ s}^{-1}$  and 0 for WT and  $\Delta flv1/3$ , respectively;  $k_{PSIFlv} = 0$  for both strains;  $k_{CBC} = 0.45 \text{ s}^{-1}$  for both strains.  $k_{CBC}$  is the rate of NADPH consumption resulting from a non or poorly activated CBC ( $k_{inact}$  in Fig. 8). It also incorporates all other processes that directly oxidize NADPH. As discussed in the main text, the lags in NADPH decay kinetics (B) are associated to the presence of  $FeS_{red}$  (A), which maintains the NADP pool to its fully reduced state. Comparison of traces a and b' of NADPH decay kinetics (B) shows that, disregarding the different lags, the NADPH decay is slightly slower in  $\Delta flv1/3$ . This small difference in simulation decays results from energetically uphill reduction of Fd by NADPH followed by  $Fd_{red}$  oxidation by Flv in WT.

**Parts C and D:** same rates as in A/B, except that  $k_{CBC} = 0.3$  and  $0.45 \text{ s}^{-1}$  for WT and  $\Delta flv1/3$ , respectively. The change in  $k_{CBC}$  rate for WT results in no visible change in the  $FeS_{red}$  decay kinetics (C). However, traces a and b' of NADPH decay (D) exhibit similar decay kinetics, contrary to B but similarly to our experimental observations (Fig. 1/A and Fig. S15). This suggests that some slow NADPH-consuming processes are a little faster in  $\Delta flv1/3$  vs WT and this effect compensates the effect of energetically uphill reduction of Fd by NADPH which occurs in WT (B)

**Parts E and F:** Flv is reduced by  $(F_A F_B)_{red}$ .  $k_{Flv} = 0$  for both strains and  $k_{PSIFlv} = 60 \text{ s}^{-1}$  and 0 for WT and  $\Delta flv1/3$ , respectively;  $k_{CBC} = 0.45 \text{ s}^{-1}$  for both strains. Traces a and b' (F) exhibit similar decay kinetics, which is attributed to the fact that reduction of  $(F_A F_B)$  by NADPH (relayed by Fd) is too energetically uphill to have an effect on NADPH decay kinetics in WT. The  $FeS_{red}$  decay kinetics in WT exhibit, as expected, a biphasic character (trace a). This character was not identified in our WT measurements, but cannot be completely excluded in view of the poor signal-to-noise ratio of the  $FeS_{red}$  signals and the possible kinetic distortions due to imperfect KLAS-NIR signal deconvolutions, as discussed in the main text.

J. Kauny, P. Sétif (2014) *Biochim. Biophys. Acta* 1837:792-801.