D1′ centers are less efficient than normal photosystem II centers

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Abstract One prominent difference between the photosystem II (PSII) reaction center protein D1′ in *Synechocystis* 6803 and normal D1 is the replacement of Phe-186 in D1 with leucine in D1′. Mutants of *Synechocystis* 6803 producing only D1′, or containing engineered D1 proteins with Phe-186 substitutions, were analyzed by 77 K fluorescence emission spectra, chlorophyll *a* fluorescence induction yield and decay kinetics, and flash-induced oxygen evolution. Compared to D1-containing PSII centers, D1′ centers exhibited a 50% reduction in variable chlorophyll *a* fluorescence yield, while the flash-induced O_2 evolution pattern was unaffected. In the F186 mutants, both the $P680^+/Q_A^-$ recombination and O_2 oscillation pattern were noticeably perturbed. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: D1; D1′; Photosystem II; *psbA*; *Synechocystis*

1. Introduction

Photosystem II (PSII) is an enzyme complex in the thylakoid membranes of chloroplasts and cyanobacteria that couples light-induced oxidation of H_2O to O_2 with reduction of quinones. The PSII reaction center core contains a heterodimer of the related D1 and D2 proteins that harbors many of the key components required for PSII photochemistry, including the reaction center chlorophyll dimer P680 (for reviews, see [1,2]). Upon light absorption, P680 is excited and transfers an electron to the primary quinone acceptor Q_A. The generated $P680^+$ is subsequently reduced by the redox-active tyrosine residue Tyr-161 on the D1 protein. Tyr-161, in turn, is reduced by electrons from H_2O, via the Mn cluster (see [3] for a recent account of the reactions on the donor side of PSII).

The D1 protein is encoded by the *psbA* gene. In chloroplasts *psbA* typically exists as a single-copy gene, whereas in cyanobacteria it belongs to small multigene families with two to six members [4–6]. In the cyanobacterium *Synechocystis* 6803 the *psbA* gene family contains three members, *psbA1*, *psbA2* and *psbA3* [4,7]. The *psbA2* and *psbA3* genes encode an identical D1 protein whereas the *psbA1* gene seems silent [4,5]. No conditions have been established under which the *psbA1* gene is transcribed. However, activation of *psbA1* by promoter engineering revealed that it encodes a novel but functional D1 protein, D1′ [8]. D1′ differs from D1 in 54 out of 360 amino acids. Three notable substitutions are a Leu for Phe at position 186 (F186L), a Ser for Pro at position 162 (P162S) and an Ala for Thr at position 286 (T286A). Phe-186 and Pro-162 are both located in the protein environment around Tyr-161, and Phe-186 has been implicated in the electron transfer reactions between Tyr-161 and P680 [9,10]. Thr-286 has been proposed to hydrogen bond to the P680 dimer [9].

Mutants of *Synechocystis* 6803 synthesizing only the D1′ protein (strain A1K), or a mixture of D1 and D1′ (strain K), have been constructed [4,8]. Both strains grow photoautotrophically with rates comparable to those of the wild type 8. To assess the activity of the D1′-containing PSII centers, the A1K and K mutants were analyzed by fluorescence and flash O_2 yield measurements and the results compared to those of control strains. Also mutants where Phe-186 in the D1 protein has been replaced with alternative amino acids residues were analyzed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Control and mutant strains of *Synechocystis* 6803 were cultured as described [4]. The incident light intensity was 50 μE m^{-2} s^{-1}. For all analyses, cells were harvested at an $OD_{730} = 0.8–1.2$ and washed in BG11 medium supplemented with 20 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.5). Cells were resuspended in the same buffer at a concentration of 0.5–0.8 mg chlorophyll ml^{-1}. The concentrated cell suspension was placed in glass tubes and kept at 30°C and 50 μE m^{-2} s^{-1} on a shaker. For fluorescence decay analyses, samples were spun down one at a time and analyses were completed within 1 h of resuspending the cells.

2.2. Chlorophyll analyses

Chlorophyll *a* concentration was determined after methanol extraction of whole cells according to Lichtenthaler et al. [11].

2.3. 77 K chlorophyll a fluorescence emission analyses

77 K fluorescence emission spectra were recorded on a Perkin Elmer luminescence spectrometer as previously described [12].

2.4. Measurements of chlorophyll a fluorescence induction and decay

Fluorescence decay measurements were performed with the Fluorescence Monitoring System FMS2 from Hansatech. Concentrated cell suspensions were diluted with BG11 plus 0.02 M HEPES–NaOH (pH 7.5) to a total amount of chlorophyll in the cuvette of

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Q_A, primary plastoquinone electron acceptor in photosystem II; Q_B, secondary plastoquinone electron acceptor in photosystem II

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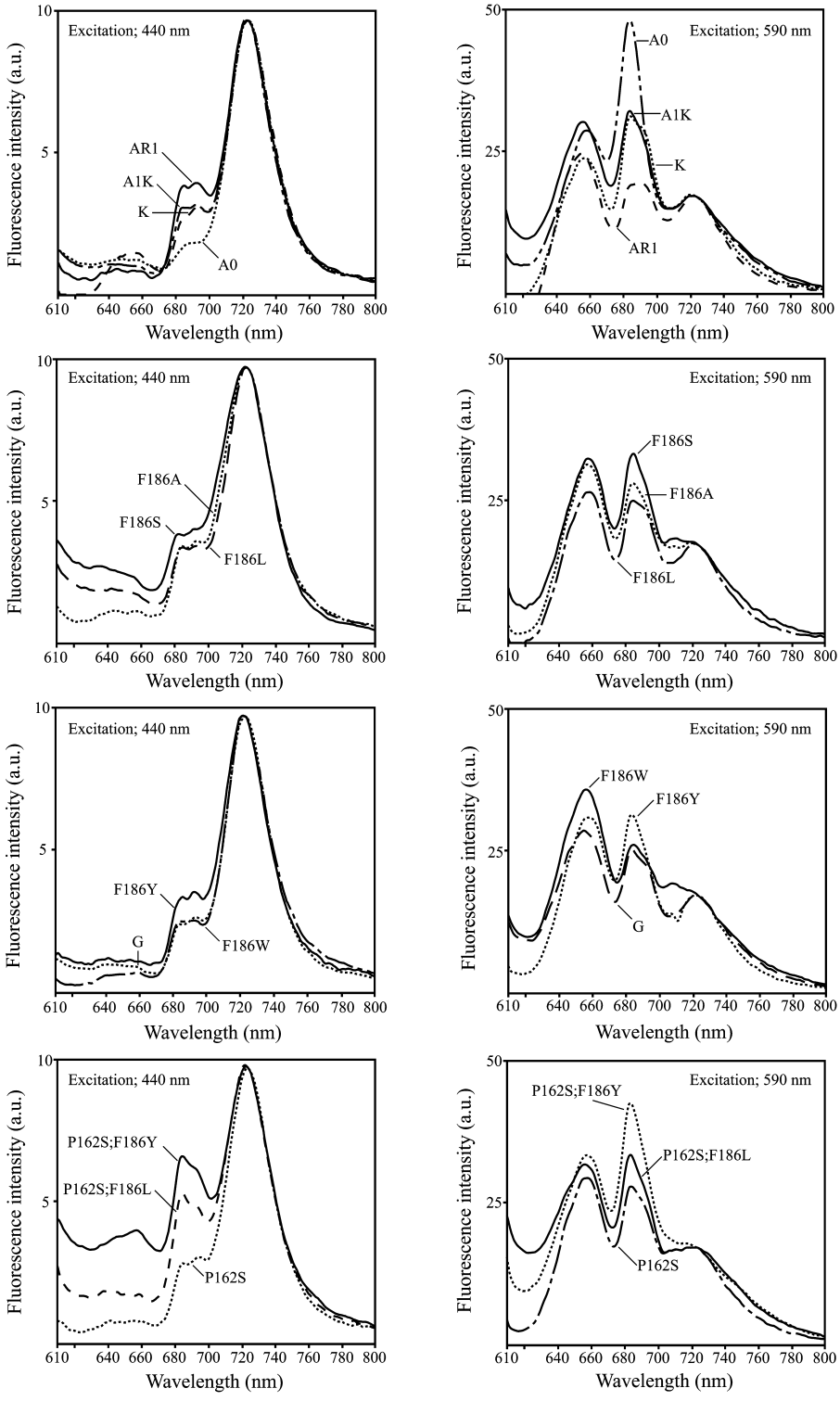

Fig. 1. Fluorescence emission spectra at 77 K from *Synechocystis* 6803 control and mutant strains after excitation at 440 or 590 nm. The spectra were normalized at 725 nm.

20 μg, corresponding to a concentration of 7 μg chlorophyll ml^{-1}. For measurements of Q_A^- charge recombination kinetics after a saturating flash, 3-(3,4-dichloro)-1,1-dimethylurea (DCMU) at a concentration of 40 μM was added to the samples in darkness 5 min before measuring.

2.5. Flash-induced oxygen evolution measurements

Flash-induced oxygen oscillation patterns were measured with a modified Joliot-type electrode [13]. The flow medium contained 0.02 M HEPES–NaOH (pH 7.5) at 25°C as described in Funk et al. [12]. The samples were dark-adapted on the electrode for 3 min, after which the polarization voltage of −700 mV was switched on for 30 s before exciting the cells with a train of short (10 μs), saturating Xe flashes separated by a dark time of 500 ms. The flash-induced oxygen yield (Y_n) was measured and normalized to the average of the yield obtained on flash 3–6, here defined as the steady-state value, Y_{ss}.

3. Results and discussion

3.1. Mutant strains

To learn about the possible functional difference between the D1 and D1′ proteins, the *Synechocystis* 6803 control strains AR1 and G [4,8] were compared with strain A0 [7] and with strains A1K, which produces only the D1′ protein, and K, which produces both D1 and D1′ [8]. Furthermore, to address the significance of the F186L substitution in D1′, a series of engineered D1 forms were produced. The properties, such as Van der Waals volume, hydrophobicity and aromaticity, of position 186 in D1 was changed by replacing the Phe with Leu, Ser, Ala, Tyr or Trp. The F186L/P162S and F186Y/P162S double substitutions were made to study the influence of the nearby Pro-162 on the residue at position 186. The construction of the various D1 forms and a general phenotypic characterization of the corresponding *Synechocystis* 6803 mutants will be presented elsewhere [31].

3.2. 77 K fluorescence emission

Measurements of 77 K fluorescence emission spectra offer a sensitive way to monitor the energy transfer to and between the photosystems, as well as the PSII/PSI ratio. Fig. 1 shows the 77 K fluorescence emission spectra for control and mutant strains after excitation at 440 nm (exciting chlorophyll *a*) or 590 nm (exciting allophycocyanin). All spectra were normalized at 725 nm, and the cells were frozen without glycerol to prevent uncoupling of the phycobilisomes from thylakoid proteins. PSI contributes to a fluorescence maximum at 725 nm, whereas maxima at 695 nm and 685 nm originate from PSII fluorescence. The main contributor to the 695 nm maximum is a low-energy chlorophyll that appears to be associated with His-114 of CP47 [14]. The rest of the PSII chlorophylls, together with allophycocyanin, are represented by the 685 nm peak. The maxima at 645 and 660 nm originate from the phycobilisome components phycocyanin and allophycocyanin, respectively [15].

The A1K and K mutants showed fluorescence emission spectra quite similar to those for the control. Both displayed peaks at 685 and 695 nm after 440 nm excitation, and a peak at 685 nm, with the shoulder at 695 nm, after 590 nm excitation. The 695 nm shoulder is less obvious in the A1K mutant, indicating a minor disturbance in PSII. In the spectra for the A0 mutant, the 695 nm fluorescence component is absent. This was expected since the A0 mutant assembles only a partial PSII complex lacking both the D1 and D2 proteins [16,17]. The prominent peak at 685 nm in the 590 nm spectrum is linked to the phycobilisomes. This spectrum resembles that of the *Synechocystis* 6803 mutant T2V;T3V;T4V, where the energy transfer between the phycobilisomes and PSII is impaired [12,18]. A similar disruption in electron transfer is likely to explain the phenotype of the A0 mutant.

For the Phe-186 and Pro-162 mutants, no drastic deviations from the control spectra were observed. Thus the energy transfer from the phycobilisomes to PSII chlorophylls and further to PSI chlorophylls seemed more or less unperturbed in the mutants.

3.3. Chlorophyll a fluorescence induction and decay

Normally, Q_A^- reoxidation is dominated by the forward electron transfer reaction to the secondary quinone acceptor, Q_B, with a $t_{1/2}$ of 100–200 μs [19], but for samples with

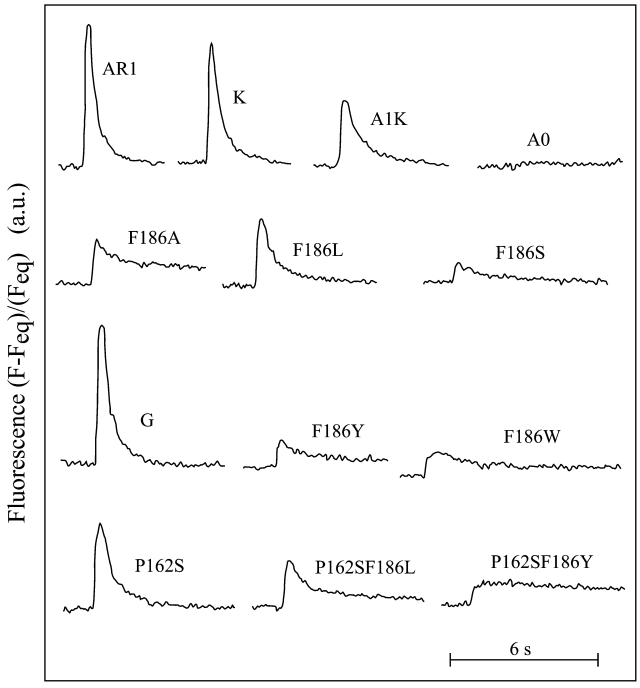

Fig. 2. Formation and decay of Q_A^- in response to a saturating flash in control and mutant strains of *Synechocystis* 6803. Measurements were performed in the presence of DCMU.

DCMU present, where the forward reaction to Q_B is blocked, the rate of Q_A^- oxidation becomes dependent on recombination reactions. In *Synechocystis* 6803 the yield of variable chlorophyll *a* fluorescence (defined as $(F_{eq}-F_o)/F_{eq}$) has been suggested to reflect the amount of Q_A^- in the sample [20] and thus this type of measurement is often used as a rapid method for quantifying PSII content [21].

The induction and decay of chlorophyll *a* fluorescence for control and mutant strains are shown in Fig. 2. The fluorescence yield for the K mutant was similar to that for the controls. For the A1K, F186L, P162S and P162S/F186L strains, the chlorophyll *a* fluorescence was reduced by approximately 50%, whereas the F186S, F186Y, F186W and P162/F186Y mutants showed virtually no chlorophyll *a* fluorescence induction. At face value, this would suggest a severe decrease in the amount of Q_A^- formation, and hence PSII complexes, in these mutants. However, as $P680^+$ and Q_A are fluorescence quenchers [22], and given the fact that position 186 is located in the vicinity of Tyr-161 and P680, the decrease in chlorophyll *a* fluorescence in the mutants could also be caused by retarded or blocked reduction of $P680^+$ by the donor Tyr-161 [22]. Since the K strain synthesizes both D1 and D1′, whereas strain A1K contains only D1′, it is likely that the better performance of the K strain, as compared to A1K, was due to the presence of the D1 protein. As predicted, no fluorescence induction was detected for the A0 mutant.

The decay kinetic from chlorophyll *a* fluorescence measurements is dependent not only on the charge recombination between Q_A^- and $P680^+$, but also on the equilibrium concentration of $P680^+$. A slower recombination kinetic would thus reflect changes on the donor side of P680. Due to the low resolution of the instrumentation used in this study, and the considerable decrease in chlorophyll *a* fluorescence in several of the mutants, a reliable decay analysis could not be per-

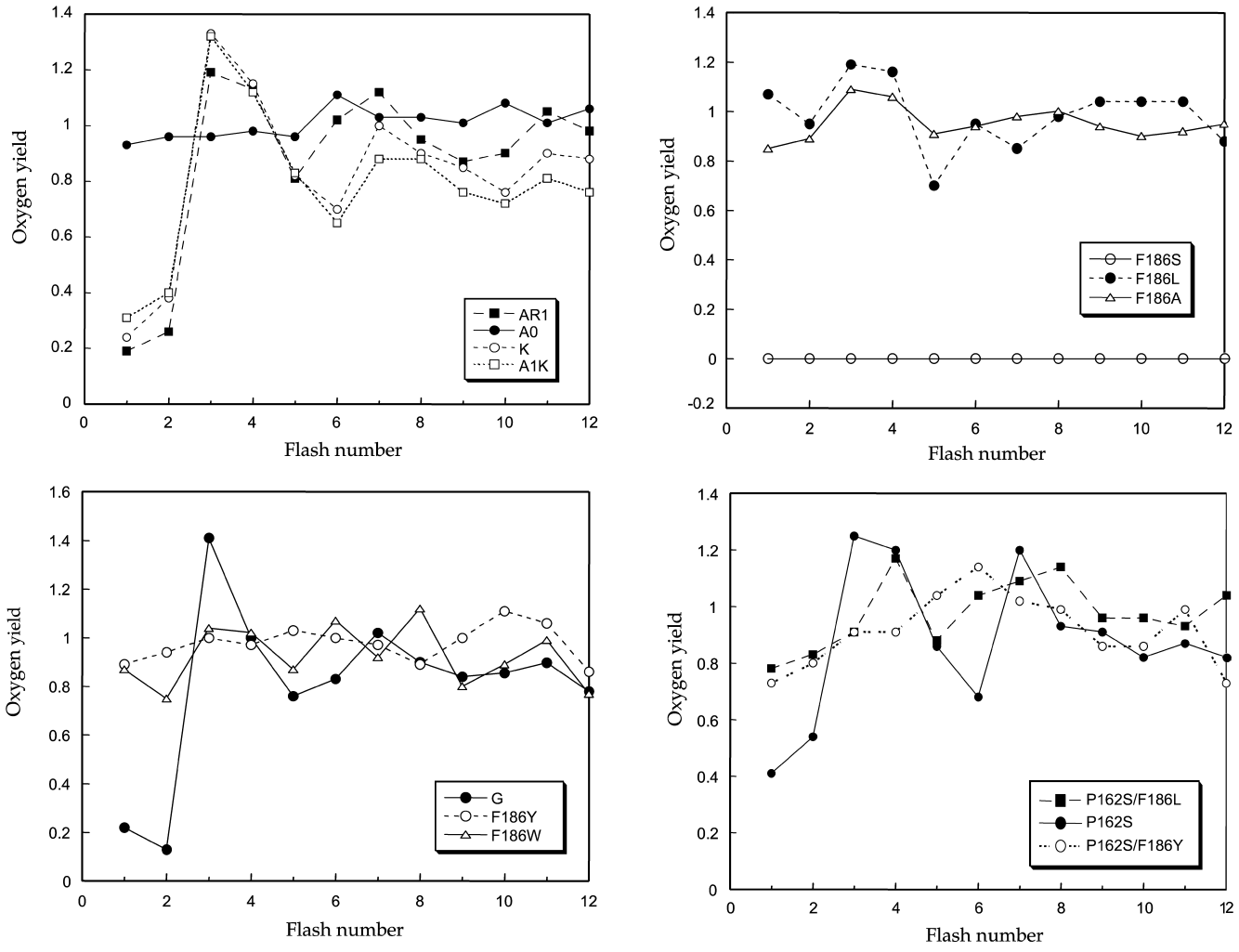

Fig. 3. Oxygen yield pattern in dark-adapted control and mutant strains of *Synechocystis* 6803, detected with a Joliot-type electrode after illumination with a series of 10 μs saturating flashes.

formed. However, a rough comparison of the decay kinetics between the different strains suggests that, compared to the controls, the K strain was not significantly affected on the donor side of P680 while the other mutants exhibited more dramatic effects.

The fluorescence characteristics of the A1K and F186 strains resemble those of the various His-190 mutants in *Synechocystis* 6803 (see [23] and references therein) and the green alga *Chlamydomonas reinhardtii* [24]. From these studies, it was concluded that the electron transfer from Tyr-161 to $P680^+$ was slowed down drastically in the His-190 mutants.

3.4. Flash-induced O_2 evolution

The O_2 oscillation pattern for intact cyanobacterial cells exhibits some differences to that of plant thylakoids [12]. Excitation of dark-adapted cyanobacterial cells with a flash train leads to a signal pattern with O_2 evolution already on the first two flashes [12,25,26]. The reasons for this phenomenon are not yet clear. Possible explanations could be an increased double hit probability in the first flashes, long-lived S_2 or S_3 states [27], or formation of H_2O_2 that interacts with the donor or acceptor side of the PSII [27,28]. Due to this 'leakage' effects in cyanobacteria, we could not reliably calculate the Kok parameters of misses and double hits. Furthermore, the partial integration of the photosynthetic and respiratory electron transport chains in the thylakoid membrane of cyanobacteria obscures interpretation of the O_2 yield.

The normalized flash patterns for control and mutant strains are shown in Fig. 3. The controls and mutants A1K and K displayed a flash pattern typical for *Synechocystis* 6803, with a fast damping oscillation of four. Also the flash O_2 yield pattern for the P162S mutant looked fairly similar to that for the controls. In contrast, all the Phe-186 mutants, as well as the A0 strain, showed a strongly disturbed O_2 evolution pattern with no periodicity. The F186S mutant produced no signals at all. At the face of it, the results in Fig. 3 indicate that all F186S mutants, including F186L, are affected in the sequential formation of S states. Probable explanations could be that the structural integrity of the Mn cluster has been perturbed or that the redox potential of the Mn cluster and/or Tyr-161 has been shifted.

3.5. Conclusion

The photoautotrophic phenotype of the A1K strain [4,8] demonstrates that the D1′ protein can replace D1 in an operational PSII complex. However, the results presented here

show that D1′ centers are less efficient than normal PSII centers, possibly due to retarded electron donation to $P680^+$. Also, the performance of the A1K strain clearly shows that Phe-186 in the D1 protein is not indispensable for PSII activity. One possible role of Phe-186 could be to contribute to the proper structural and hydrophobic environment around the redox-active Tyr-161, to ensure fast electron donation to $P680^+$. The hydrophobicity and Van der Waals volume are similar for Phe and Leu, which might be the reason for the less drastic phenotype of the F186L strain, as compared to the other Phe-186 mutants. However, the flash oxygen yield measurements revealed that the F186L substitution alone cannot explain the functional S cycle observed in the A1K strain. Possibly, the accumulated effect of the amino acid substitutions in the vicinity of P680 in the D1′ protein [8] is compatible with normal S cycle operation, whereas the single F186L or the double F186L/P162S substitutions are not.

It should be pointed out that D1′ in *Synechocystis* 6803 is not related to D1:2 in *Synechococcus* 7942, which is transiently produced under stress conditions [29,30]. The presence of the *psbA1* gene in *Synechocystis* 6803 is enigmatic [5,8], and whether or not the D1′ protein serves a function in the *Synechocystis* 6803 cells under specific environmental conditions is still an open question.

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