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# The slow S to M fluorescence rise in cyanobacteria is due to a state 2 to state 1 transition ☆

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ABSTRACT

In dark-adapted plants and algae, chlorophyll *a* fluorescence induction peaks within 1 s after irradiation due to well documented photochemical and non-photochemical processes. Here we show that the much slower fluorescence rise in cyanobacteria (the so-called "S to M rise" in tens of seconds) is due to state 2 to state 1 transition. This has been demonstrated in particular for *Synechocystis* PCC6803, using its RpaC$^-$ mutant (locked in state 1) and its wild-type cells kept in hyperosmotic suspension (locked in state 2). In both cases, the inhibition of state changes correlates with the disappearance of the S to M fluorescence rise, confirming its assignment to the state 2 to state 1 transition. The general physiological relevance of the SM rise is supported by its occurrence in several cyanobacterial strains: *Synechococcus* (PCC 7942, WH 5701) and diazotrophic single cell cyanobacterium (*Cyanothece* sp. ATCC 51142). We also show here that the SM fluorescence rise, and also the state transition changes are less prominent in filamentous diazotrophic cyanobacterium *Nostoc* sp. (PCC 7120) and absent in phycobilisome-less cyanobacterium *Prochlorococcus marinus* PCC 9511. Surprisingly, it is also absent in the phycobiliprotein rod containing *Acaryochloris marina* (MBIC 11017). All these results show that the S to M fluorescence rise reflects state 2 to state 1 transition in cyanobacteria with phycobilisomes formed by rods and core parts. We show that the pronounced SM fluorescence rise may reflect a protective mechanism for excess energy dissipation in those cyanobacteria (*e.g.* in *Synechococcus* PCC 7942) that are less efficient in other protective mechanisms, such as blue light induced non-photochemical quenching. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial Photosynthesis.



## 1. Introduction

Chlorophyll a (Chl a) fluorescence is a powerful, non-invasive and sensitive tool to monitor photosynthesis [1–3]. It reflects processes with various kinetics starting from ultrafast excitation energy transfer in light-harvesting antennae [4] followed by charge separation in reaction centers [5,6] and slower electron and proton transport processes in photosystems [7] including movement of protein complexes [8,9]. The fast Chl *a* fluorescence induction, observed from μs to s range, and labeled as OJIP transient (O for the initial minimum fluorescence, J and I for inflections, and P for the peak), is mostly related to the electron transport in the thylakoid membrane [10–12]. The slower (tens of seconds) SM fluorescence rise (with M representing a second maximum) is usually dominant in phycobilisome (PBS)-containing cyanobacteria [13–15]. Since the SM rise occurs also in the presence of DCMU, involvement of electron transport between $Q_A$ and $Q_B$ and the plastoquinone pool (PQ-pool) has been ruled out [16]. Thus, the SM rise in cyanobacteria must reflect a regulatory distribution of light excitation energy from the less fluorescent photosystem I (PSI) to more fluorescent photosystem II (PSII) — an involvement of state 2 to state 1 transition in this process was suggested [13,14].

*Abbreviations:* APC, allophycocyanin; Chl *a*, chlorophyll *a*; FI, fluorescence induction; $F_m$ ($F_m$'), maximal chlorophyll a fluorescence for dark (light) adapted sample, $\Delta F_m$'(rel), relative changes in maximal fluorescence in light (in %); PC, phycocyanin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NPQ, non-photochemical quenching of fluorescence; OCP, Orange Carotenoid Protein; PAR, Photosynthetically Active Radiation; PBS, phycobilisome; PQ, plastoquinone; PS I (PS II), photosystem I (photosystem II); RpaC$^-$, mutant cells lacking the protein described as "Regulator of Phycobilisome Association, C"; RT, room temperature; WT, wild type cells; SRFI, Spectrally resolved fluorescence induction

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There is no consensus for the mechanism of state transitions in cyanobacteria (for reviews, see *e.g.* [17,18]) and also in phycobiliprotein containing red algae [19,20]. In all oxygenic photosynthetic organisms, state transitions are initiated by redox shifts in the PQ-pool; transition to state 2 is triggered by its reduction, transition to state 1 starts with its oxidation [8,21,22]. In contrast to plants and green algae, no protein phosphorylation is involved in state transitions in cyanobacteria. Essentially, two mechanisms of state transitions have been identified in cyanobacteria [8,23–26]. In the first mechanism, physical movement of PBS [24] or PSI monomers [27] leads to redistribution of energy absorbed in PBS between PSII and PSI. In the second mechanism, excitation energy spillover [21,23] regulates the redistribution of light absorbed by Chl *a*, between PS II and PSI. It is generally assumed that state transition mechanism requires some movement of membrane complexes and/or changes in their oligomerization state [28]. However, it is not clear if long-range displacement of proteins (PBS, PSI) is necessary for state changes *in vivo* [24,26] or whether only a slight protein movement is enough to explain the observed results [23]. The complete model of state transition must also take into account the organization of protein supercomplexes in thylakoid membranes [29,30]. We know that in state 1, a higher percentage of PS II complexes are associated in rows in contrast to their rather random distribution in state 2 [31,32]. Also, PSI oligomerization changes with state transitions as the presence of PSI in trimers is more typical for state 2 [33]. Surprisingly, such PSI monomerization is accompanied by higher mobility of PBS [34], but its effect on the mobility of PSI remains a speculation [27], since mobility of large PSI complexes is difficult to measure [9]. Therefore, an influence of the oligomerization form of PSI on the process of state transition remains to be solved [35,36], especially as it is significant during the day cycle of diazotrophic cyanobacteria [37,38]. In summary, despite decades of research on state transitions in cyanobacteria, the underlying mechanism remains still unresolved including the question of how changes in the plastoquinone redox state trigger the observed changes in energy redistribution between PSI and PSII.

In the present work, we have established that the slow SM rise of Chl *a* fluorescence, caused by excitation of phycobilins with high intensity orange light, is due to the state 2 to state 1 transition. Our conclusion is based on the absence of SM rise in both state 1-locked cells (mutant of *Synechocystis* PCC 6803 without RpaC$^-$ protein [39]) and in state 2-locked cells (in hyper-osmotic suspension [40]) of wild-type *Synechocystis* PCC 6803. Therefore, the SM rise of Chl a fluorescence can be considered as a marker of the state 2 to 1 transition in *Synechocystis* sp. PCC 6803. We have also shown different manifestations of the SM rise in various cyanobacterial species: these were detected only in cyanobacteria with PBS that consist of both phycobiliprotein rods and cores, namely in *Synechocystis, Synechococcus* (PCC 7942, WH 5701) and in the diazotrophic cyanobacterium (*Cyanothece* sp. ATCC 51142). However, it was absent in phycobiliprotein rod containing *Acaryochloris marina* (MBIC 11017). We suggest that the pronounced SM fluorescence rise reflects a safe way of excitation energy dissipation after a sudden exposure to excessive light, as it is most dominant in cyanobacteria (*e.g.* in *Synechococcus* PCC 7942) that lack other protective mechanisms like the blue light induced non-photochemical quenching.

## 2. Materials and methods

### 2.1. Photosynthetic organisms

Freshwater cyanobacteria *Synechocystis* (Pasteur Culture Collection, PCC 6803) and *Synechococcus* sp. (Pasteur Culture Collection, PCC 7942) were cultivated in BG 11 medium [41], in an orbital shaking incubator, at 28 °C and at a constant irradiance of 14 μmol photons $m^{-2}$ $s^{-1}$ of PAR (Photosynthetic Active Radiation, 400–700 nm). A mutant of *Synechocystis*, RpaC$^-$ (lacking the protein described as "Regulator of PBS Association — C") was obtained from Conrad Mullineaux (Queen Mary College, London, UK). Cells for measurements were collected 5 days after inoculation in the exponential growth phase ([Chl]~1.5–1.8 μg Chl *a*/ml).

Marine cyanobacteria *Synechococcus* sp. (WH 5701) and *Prochlorococcus marinus* (PCC 9511) were cultivated at a constant irradiance of 15 μmol photons $m^{-2}$ $s^{-1}$ (t = 20 °C) in PCR-S11 [42] medium without shaking. Diazotrophic cyanobacteria *Nostoc* sp. (PCC 7120, formerly known as *Anabaena* sp.) and *Cyanothece* sp. (ATCC 51142) were grown in BG 11 [41] and ASP II [43] media respectively, with air bubbling, in a sinusoidal light:dark cycle (day night 12/12, maximum 30 μmol photons $m^{-2}$ $s^{-1}$) at 28 °C. The cyanobacterium *Acaryochloris marina* (MBIC 11017) was cultivated at a constant irradiance of 15 μmol photons $m^{-2}$ $s^{-1}$ (t = 20 °C) in YBC-II medium [44] without any shaking. Thermophilic cyanobacterium *Synechococcus bigranulatus (*KOVROV 1972/8) [45] was cultivated in continuous light in tubes bubbled with $CO_2$ enriched air (2% of $CO_2$) at 58 °C in a medium [46] that was modified by the addition of 10 mM $NaHCO_3^-$.

### 2.2. Low temperature fluorescence spectroscopy

Low temperature fluorescence spectra were measured in liquid nitrogen (77 K) with a SM-9000 spectrophotometer (Photon Systems Instruments, Czech Republic), using 530 nm excitation light (spectral bandwidth of excitation = 20 nm; and of emission = 0.8 nm). Samples were collected either in dark or after exposure to orange actinic light (590 nm, 300 μmol photons $m^{-2}$ $s^{-1}$; 200 s), the particular spectra were de-convoluted by Origin Pro "Peak Analyzer", using Gaussian curves. During the fitting procedure, only positions of peak maxima were restricted to 8 nm range, around the predicted maxima; all other parameters were set to be free for the minimization procedure, driven by Chi-square (with $10^{-6}$ precision).

### 2.3. Chlorophyll a fluorescence induction

Chlorophyll *a* fluorescence induction (FI) kinetics were measured with a FL-100 Fluorometer (Photon Systems Instruments, Czech Republic) with orange light (590 nm; Δλ, 20 nm; 300 μmol photons $m^{-2}$ $s^{-1}$) excitation. Fluorescence induction was measured in the 690–750 nm range. Samples were dark adapted for 20 min before measurements. To obtain maximal fluorescence, $F_m$, that reflects full closure of the PSII reaction centers, a saturation flash was applied (590 nm; Δt 200 ms; ~1200 μmol photons $m^{-2}$ $s^{-1}$). Subsequently, variable fluorescence kinetics, induced by orange actinic irradiation, was measured during 10 min with logarithmically increasing intervals between the measured points (55 points per decade, first point was 26 μs after the light onset).

### 2.4. Spectrally resolved fluorescence induction (SRFI)

Fluorescence emission spectra during Fluorescence Induction (FI) were measured by the Spectrally Resolved Fluorescence Induction (SFRI) method) [47] with a protocol that allowed detection of differences in the spectrum of $F_m$' [48]. Actinic and saturating illumination of the samples was as described in Section 2.3. Emission spectra (600–800 nm) were recorded every 90 ms with SM-9000 spectrophotometer (Photon Systems Instruments, Czech Republic) that was constructed from Carl-Zeiss spectrometer module "MCS CCD UV-NIR 200-980" (Carl Zeiss Jena GmbH, Germany) equipped with CCD array detector (absolute wavelength accuracy of 0.8 nm; relative resolution reflecting FWHM Δλ = 3 nm) produced by Hamamatsu Photonics (Hamamatsu City, Japan). The measured two-dimensional data were processed by Origin Pro software (version 8.5, Origin Lab Corp., USA).

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### 2.5. Blue light induced non-photochemical quenching (NPQ)

Blue light induced non-photochemical quenching was measured based on a typical quenching analysis (see *e.g.* [3]) for cyanobacteria. Maximal fluorescence ($F_m$) was measured for a dark-adapted sample with low intensity blue light (15 μmol photons $m^{-2}$ $s^{-1}$, 464 nm, Δλ ~ 20 nm) on background to put the cells into State 1 and maximal fluorescence of dark-adapted cells in State 1 was then detected during saturation flash. Non-photochemical quenching (NPQ) was induced by a 6-minute long irradiation by strong blue light (1200 μmol photons $m^{-2}$ $s^{-1}$, 464 nm, Δλ ~ 20 nm); after this period, maximal fluorescence in light ($F_m$') was measured during 200 ms long saturation flash (see *e.g.* [49]). The NPQ value was calculated using Stern–Volmer formula as NPQ = ($F_m - F_m$')/$F_m$', the relative changes in maximal fluorescence after irradiation (in percentage) were characterized by parameter of maximal fluorescence increase on light $\Delta F_m'(rel) = 100 * (F_m' - F_m)/F_m$.

## 3. Results

### 3.1. 77 K emission spectra of wild type and RpaC$^-$ mutant cells

A comparison of 77 K fluorescence emission spectra of *Synechocystis* sp. PCC 6803 of RpaC$^-$ mutant cells with that of WT cells shows that the RpaC$^-$ mutant is locked in the high fluorescent state 1 since the ratio of PSII (F684, F692) to PSI (F726) fluorescence emissions is higher in comparison to WT cells (Figs. 1 and 2). This is in agreement with earlier conclusions [39]. The origins of the three Chl *a* fluorescence bands, observed at 77 K (Fig. 1) are: F684 and F692 are emitted by Chls *a* of the PS II core antennae CP43 and CP47, respectively (see *e.g.*, [50] for a review), and F726 by Chls *a* of PSI. Following illumination with 590 nm light (absorbed by phycobilins), untreated WT cells show a slight increase of PSII emission bands in comparison to their PSI band (Fig. 1A, *untreated*); in DCMU-treated cells, the increase in the emission bands of PSII, in comparison to the PSI band, was much larger (Fig. 1A, *+DCMU* curve). Since we used 590 nm light for excitation, the changes in F692/F726, following actinic irradiation, are explained as a result of state 2 to state 1 transition — while quenching by the orange carotenoid protein [51,52] is expected to be insignificant.

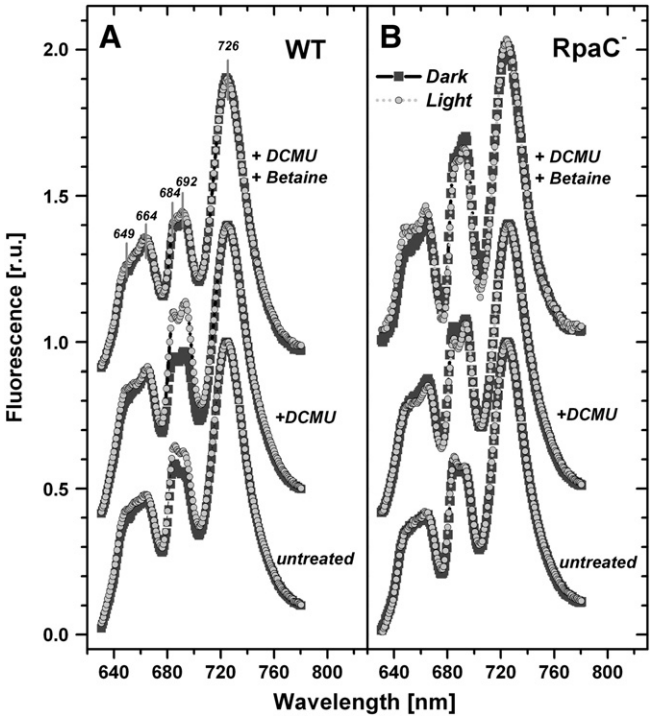


**Fig. 1.** Low temperature (77 K) fluorescence spectra of wild type (WT) cells (A) and RpaC$^-$ mutant cells (B) of *Synechocystis* sp. *PCC 6803*. Spectra were recorded either with dark-adapted cells (20 min, black circles), or with light-adapted cells (200 s; orange light 590 nm, 300 μmol photons $m^{-2}$ $s^{-1}$; gray circles). Fluorescence excitation, $\lambda_e = 530$ nm, $\Delta\lambda = \pm 20$ nm (green diode); fluorescence detection, $\lambda_f = 630$–780 nm; $\Delta\lambda = 0.8$ nm. Chemicals, when present, were added before the measurements: DCMU (10 μM); DCMU (10 μM) with glycine betaine (*Betaine*, 500 mM). Data are normalized to fluorescence at 726 nm; the positions of particular emission maxima are marked only in the top curve in panel A.

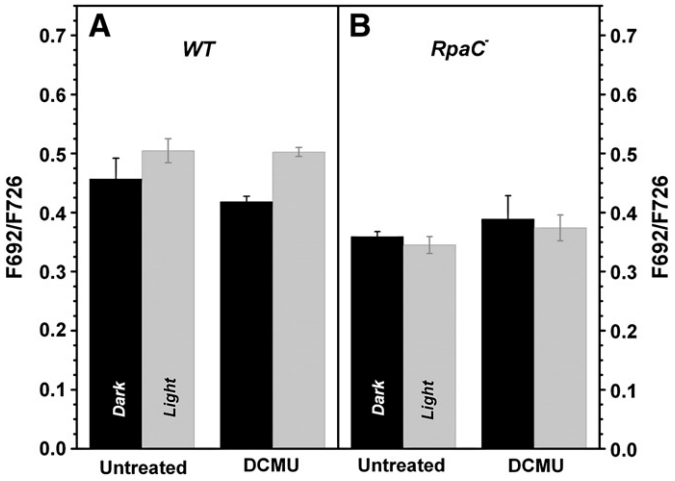


**Fig. 2.** The ratio of the 77 K fluorescence maxima, F692/F726 (Photosystem II/Photosystem I), measured with WT (A) and RpaC$^-$ mutant (B) of *Synechocystis* sp. *PCC 6803*. Fluorescence excitation, $\lambda_e = 530$ nm, $\Delta\lambda = \pm 20$ nm (green diode), standard deviation bars are for n = 3. Ratios were obtained after curve deconvolution; other details are as in Fig. 1 legend.

Fig. 2 shows the F692/F726 of Chl *a* fluorescence at 77 K, calculated from the data in Fig. 1. It shows, as mentioned above, a higher value of this ratio in state 1, and a lower value in state 2 cells. Thus, the observed relative increase is due to the state 2 to state 1 transition (see Fig. 2). Actinic irradiation caused about 10% increase in the F692/F726 ratio in untreated WT cells, and about 30% increase in the presence of DCMU (Fig. 2A). No increase was observed in the F692/F726 ratio of the RpaC$^-$ mutant cells upon actinic illumination (see Figs. 1B and 2B, *untreated* and *+DCMU* curves), providing evidence that these cells are locked in high fluorescent state 1. In contrast, WT cells in hyperosmotic suspensions (in the presence of glycine betaine) were locked in the low fluorescence state 2 (see Fig. 1A, *+DCMU + Gl. betaine* curves) because hyperosmotic conditions shift cells to the low fluorescence state 2 [53,54]. In contrast, the RpaC$^-$ mutant was in high-fluorescent state 1 even after betaine addition (see F692 band in Fig. 1B), suggesting that PBS are so tightly bound to PS II (locked in state 1) that even hyperosmotic conditions cannot shift them to state 2.

### 3.2. The SM fluorescence rise is due to state 2 to state 1 transition

Fig. 3 shows Chl *a* FI patterns of WT (panel A) and of RpaC$^-$ mutant cells (panel B) of *Synechocystis* sp. PCC 6803. The fast OJIP fluorescence rise of untreated WT cells peaks at P at ~1 s and from there, fluorescence declines by ~15%. In contrast to the well-known FI pattern of *Synechococcus* sp. PCC 7942 cells, which consists of a low amplitude fast transient (OJIP) and a high amplitude slow fluorescence transient (SMT; see *e.g.* [13,47]), the FI pattern of untreated *Synechocystis* cells (Fig. 3A) shows no clear features after P that can be labeled as S or M. However, in the presence of DCMU, which closes the PSII reaction centers, *Synechocystis* shows clearly both the low amplitude fast OPS transient and the high amplitude slow SMT

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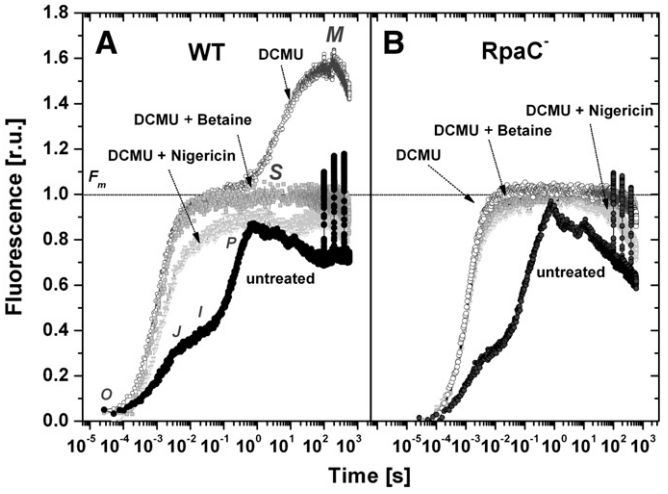


**Fig. 3.** Time course of chlorophyll *a* fluorescence measured with WT (A) or RpaC$^-$ mutant (B) of *Synechocystis* sp. PCC 6803. Samples without any addition (*untreated*) or in the presence (where indicated) of DCMU (10 μM), nigericin (60 μM) or glycine betaine (500 mM). Data are normalized to the maximal fluorescence obtained after the samples were excited with a saturating light pulse given just before the fluorescence time course was recorded. Three saturation pulses were applied at 200 s, 430 s and 600 s to obtain $F_m'$ (maximum fluorescence in light). Characteristic inflections and peaks (O–J–I–P–S–M) during the fluorescence transient are marked in two of the curves of panel A. Fluorescence excitation, $\lambda_e = 590$ nm, $\Delta\lambda = \pm 20$ nm (amber diode).

transient of FI. The slow fluorescence transient is seen also in the WT cells when PSII reaction center is closed by saturating actinic light pulses (*untreated*, Fig. 3A). We show, further, that the slow Chl *a* fluorescence rise along SM, in the case of DCMU-treated WT cells, is blocked by the uncoupler nigericin, as well as by the addition of hyper-osmotic concentrations of glycine betaine (Fig. 3A).

Fig. 3B shows the FI patterns of RpaC$^-$ mutant cells. Since these cells are blocked in state 1 (see Fig. 2), the fast transient, observed with untreated cells, must correspond to a Chl *a* fluorescence rise along OJIP. It is likely, however, that the latter peak amplitude includes both the P and the M contributions. This may explain the steeper decline of Chl *a* fluorescence in the case of RpaC$^-$ cells (Fig. 3B, untreated) compared to the WT cells (Fig. 3A, untreated). In the presence of DCMU (±nigericin; or ±glycine betaine), the RpaC$^-$ cells, being already in state 1, show no further rise of Chl *a* fluorescence. All the above results clearly prove that the SM rise corresponds to a state 2 to state 1 transition of *Synechocystis* cells, because (a) it is absent in the WT cells that are locked in state 2 by nigericin or by hyperosmotic glycine betaine; and (b) it is absent in the state 1-locked RpaC$^-$ mutant.

### 3.3. Fluorescence induction: room temperature measurements at several emission wavelengths

State transitions were further explored by spectrally resolved fluorescence induction method [47]. This method involves time-dependent spectral fluorescence measurements of all the emitting chromophores (see Fig. 4 for *Synechocystis* sp. PCC 6803 with DCMU). The panel labeled *Spectrally Resolved Fluorescence Induction (SRFI)* displays graphically the emitted fluorescence intensity $F_{t,\lambda}$ (dependent variable) as a function of the fluorescence wavelength (λ; x-axis) and the duration of the illumination (t; y-axis). The fluorescence intensity is plotted in color-coded intensity scale (low fluorescence in violet and blue, high fluorescence in red) together with isoemissive contour lines in black. In this way, a section parallel to the wavelength axis (x-axis) represents the fluorescence emission spectrum at a given time of irradiation (see top left panel labeled as *Spectrum*, Fig. 4), while a section parallel to the time axis (y-axis) yields the fluorescence induction time course at a particular wavelength (see top right panel labeled as *Fl. Induction*, Fig.4). Data presented in Fig. 4 show an example of 2D curve obtained by the SRFI method for *Synechocystis* sp. (in this case treated with DCMU). We can see that the dominant fluorescence emission is situated in the 660–690 nm region; this is due to emission of both phycobiliproteins and PSII Chls.

The SRFI method provided information on: (1) changes in fluorescence emission spectra at RT during irradiation (*i.e.*, at different portions of the FI curves) for *Synechocystis* sp. PCC 6803 WT and its RpaC$^-$ mutant (Fig. 5); and (2) fluorescence induction at a single wavelength (F682 in Fig. 6).

The room temperature spectra of WT and RpaC$^-$ mutant of *Synechocystis* PCC 6803 show three characteristic maxima (Fig. 5) in line with previous results (see e.g. [55]) and that can be interpreted as follows: (1) F658 — APC emission; (2) F682 — emission due to Chls *a* of the PS II core complexes (PS II-Chl ) as well as APC-B, an APC connected to the terminal APC emitter (APC-$L_{cm}$) (3) F720 — a broad and low maximum mainly due to emission from Chls a of PS I (PS I–Chl). There were no significant changes in the positions of these bands during irradiation, or due to DCMU addition in both the WT and the RpaC$^-$ cells (see Fig. 5, A–D). The most visible change in the room temperature spectra was in the relatively pronounced increase of F682 (emitted by PSII Chl *a* plus a minor contribution by APC) during the first second of irradiation and only very small changes in APC emission at 658 nm (Fig. 5A and B). These changes can be related to the fast spectral changes during the IP phase of fluorescence induction (Fig. 3). However, spectral changes in the slower P to S (and M) phases of fluorescence induction are shown by "70 s" and "250 s" curves (Fig. 5). There, F682 band slightly increased in WT and decreased in RpaC$^-$ mutant of *Synechocystis* PCC 6803 between 70 s and 250 s (compare Fig. 5A with Fig. 5B). These changes in F682 were inhibited in RpaC$^-$ in the presence of DCMU (Fig. 5D).

Spectrally resolved fluorescence induction (SRFI) at room temperature allowed us to observe these small changes in the intensity of F682 in details (Fig. 6A). After irradiation of untreated cells, an increase in F682 during the SM phase was detectable in the WT cells; however, it was relatively small (from 0.88 to 0.92) after 60 s to 300 s of irradiation. A similar increase in F682 was clearly absent in RpaC$^-$ mutant cells; instead a decrease was observed (Fig. 6A). In WT cells, with reaction centers closed by DCMU, the SM rise was much higher and faster as it started already after 1 s of irradiation (Fig. 6B; also see Fig. 3A). This SM rise of F682 was inhibited by hyperosmotic suspension (in the presence of the non-penetrating osmolyte glycine betaine), which locks the cells in the low fluorescence state 2. Therefore, the slow F682 rise after irradiation is due to state 1 to state 2 transition in line with the data presented in Fig. 3; thus, such an increase in F682 was missing in the RpaC$^-$ mutant (Fig. 6A) confirming its inability to perform state changes [39].

Since the slow fluorescence rise was more pronounced when the PSII reaction centers were closed, either after saturating excitation flashes (Fig. 3) or in the presence of DCMU (Figs. 3 and 6), we further explored the changes in fluorescence emission of closed reaction centers at the $F_m$' state (see Fig. 4, top left curve). Fig. 7 shows the differences observed in the emission spectra of the WT and RpaC$^-$ mutant induced by actinic irradiation. As expected for the state 2 to state 1 transition in the WT cells, the F682 emission at 600 s increased relative to the level at 60 s. On the other hand, F682 decreased in the RpaC$^-$ mutant cells, suggesting that either quenching or photoinhibition occurs in the absence of state changes. Deconvolution of spectral changes induced by state transition in *Synechocystis* PCC 6803 WT (Fig. 7A) compared to the quenching observed in the RpaC$^-$ mutant (Fig. 7B) showed the presence of four main bands (data not shown): ~F662 (PC emission), ~F682 (due to both PSII Chl a and APC emission), ~F700 (PSI Chl a emission), F735 (vibration satellite of PSII Chl a emission). In both cases, maximum changes in the difference spectrum were

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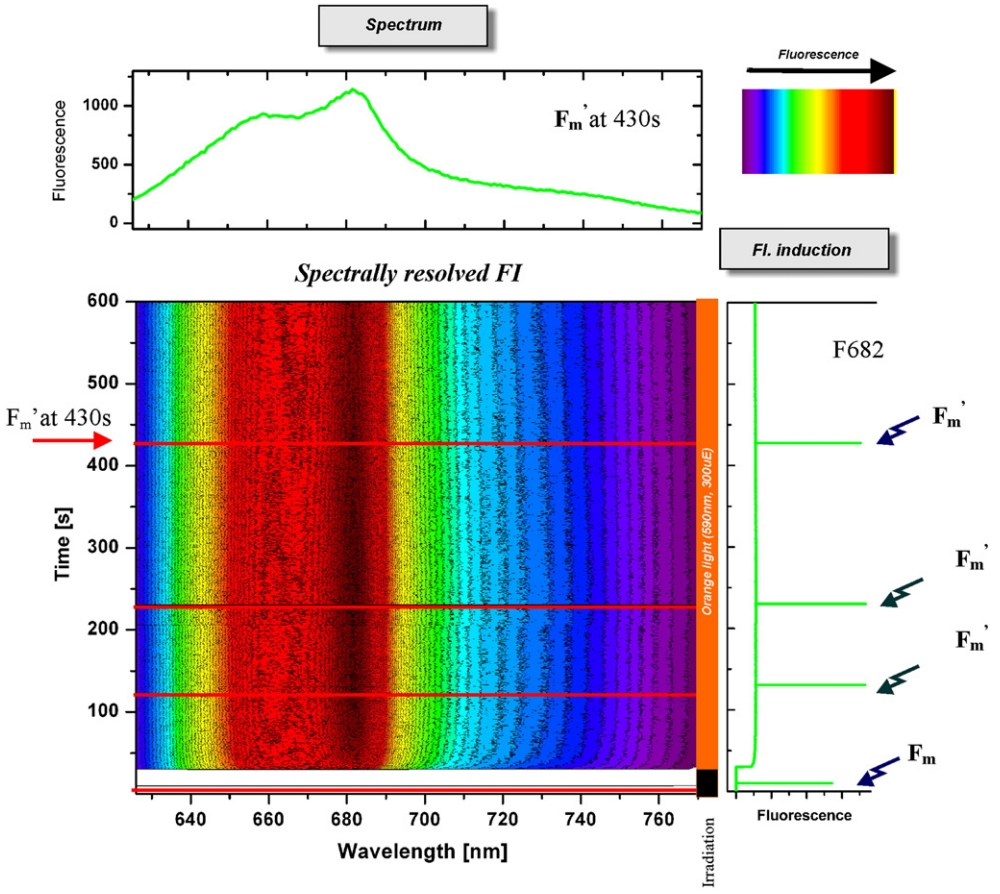


**Fig. 4.** A typical two dimensional (2D) plot of Spectrally Resolved Fluorescence Induction (SRFI) of WT *Synechocystis* sp. PCC 6803 in the presence of 10 μM DCMU. For this plot, raw fluorescence induction data were recorded with 20 min dark-adapted cells at room temperature. Fluorescence excitation, $\lambda_e = 590$ nm, 300 μmol photons $m^{-2}$ $s^{-1}$, $\Delta\lambda = 20$ nm. The graph shows $F = f(\lambda, t)$, where one dependent variable F (fluorescence intensity; color-coded isoemissive contours are plotted against two independent variables (wavelength ($\lambda$) in the range of 620 to 800 nm (the x-axis); and the time of illumination, t(s), in the range of 0–600 s; the y-axis). The entire spectrum was measured every 100 ms. Panel labeled as *Spectrum* (top, left) displays a section, parallel to the wavelength axis, at 200 s, whereas the panel *Fl. Induction* (right) displays fluorescence induction at selected wavelength (at 682 nm).

centered at 682 nm (combined emission of PSII Chls *a* and of APC). The F662 emission was dominant in RpaC$^-$ mutant, but was small in WT.

We also measured reversibility of the observed changes in dark (see "60 s recovery" curves in Fig. 7); in both WT and RpaC$^-$, the difference spectra decreased to about half following the dark period (Fig. 7). We suggest that the inability to dissipate excess PS II excitation by means of a state 1 to state 2 transition forces the RpaC$^-$ mutant to adopt another mechanism: reversible quenching of excitation (with maxima at 665 nm and at 682 nm). This supports the hypothesis of co-operativity between the state transition changes and the non-photochemical quenching (NPQ) in the maintenance of redox balance in plants [56].

### 3.4. Species variability in the S to M fluorescence rise reflects state changes at high irradiance

The presence, or the absence, of the S to M fluorescence rise and its significance for state transitions was further studied in representative cyanobacterial species from various clades by fluorescence induction (Fig. 8). We used *Synechococcus* species from marine (WH 5701) and fresh water (PCC 7942) environment, diazotrophic ($N_2$-fixing) cyanobacteria, either single celled (*Cyanothece* sp. — PCC 7120) or filamentous (*Nostoc* sp. MBIC 11017). We also made measurements on cyanobacteria that lack the classical PBS – either having only limited PBS rods together with Chl *a*/*d* proteins – *Acaryochloris marina* (MBIC 11017) [57] or cyanobacteria that lack extrinsic functional phycobiliproteins altogether — *Prochlorococcus* (PCC 9511), but have instead intrinsic divinyl-Chl *a*/*b* antennae [58,59].

We observed a pronounced SM fluorescence rise in *Synechococcus* strains (PCC 7942 and WH 5701) in which the M-level fluorescence in the presence of DCMU (that stimulates the state 2 to state 1 transition) was more than three times higher in light in comparison to the dark $F_m$ values (see Figs. 8A and B). The SM rise was less prominent in the filamentous diazotroph *Nostoc* sp. (Fig. 8C), but quite prominent in the single-celled diazotroph *Cyanothece* sp. (Fig. 8D). In contrast, *Acaryochloris marina* that has phycobiliprotein rods [60], but lacks complete PBS, and *Prochlorococcus* sp., that lacks phycobiliproteins altogether but has instead intrinsic divinyl-Chl *a*/*b* as light-harvesting antennae, did not show any SM rise in Chl *a* fluorescence transient, either with or without DCMU (Fig. 8E and F). This agrees with previously reported results [13], which showed that the S to M fluorescence rise requires the presence of complete PBS that comprise both PC rods and APC cores. The S to M fluorescence rise was also inhibited by hyperosmosis (induced by glycine betaine) that locks cells in the weak fluorescence state 2 (Fig. 8). As noted above, this effect was clearly visible in freshwater *Synechococcus* PCC 7942 (Fig. 8A). In marine cyanobacteria with PBS *Synechococcus* WH 5701 and *Cyanothece* ATCC 51142 (see Fig. 8B and D), the effect of glycine betaine was probably also influenced by their adaptation to hyperosmosis due to the presence of channels that are necessary for balancing hyperosmosis in marine species [61].

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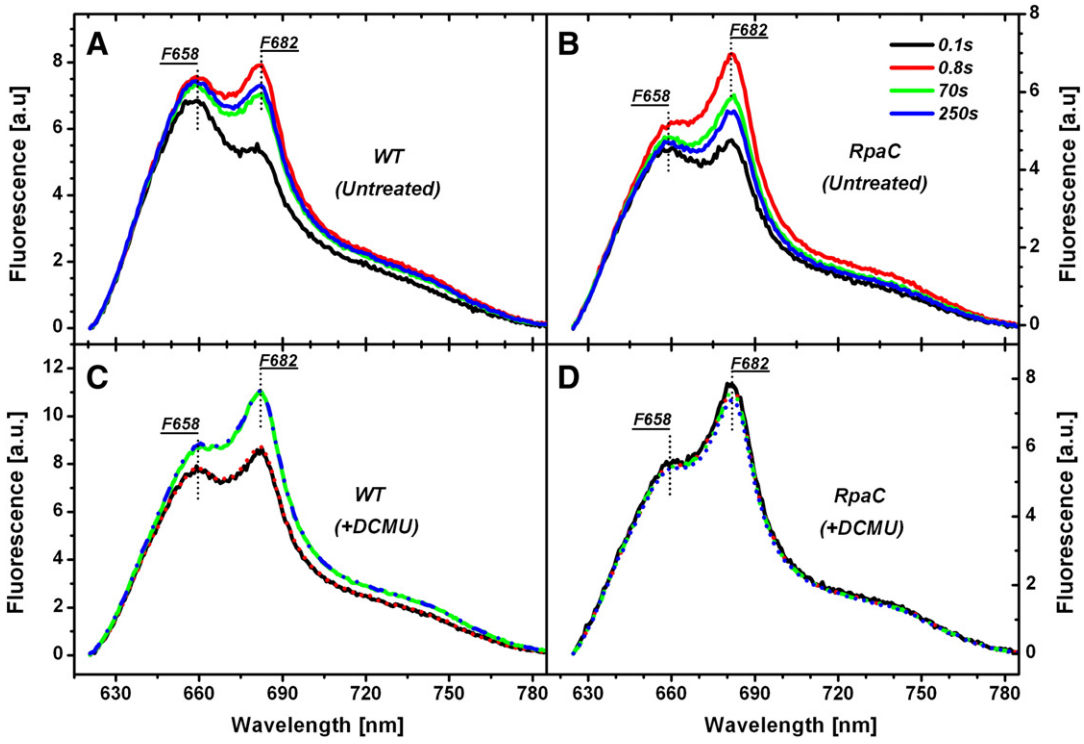


**Fig. 5.** Room temperature fluorescence spectra at specific times of the fluorescence induction curves of WT (A and C) and RpaC$^-$ mutant (B and D) of *Synechocystis* sp. PCC 6803. The spectra were measured by SRFI method at selected times (corresponding phases of the fluorescence induction, see Fig. 3): 0.1 s (I to P in $-$*DCMU* cells;); 0.8 s (P level in $\pm$*DCMU* cells); 70 s (P to S in $-$*DCMU* cells, nearly M in $+$*DCMU* cells); 250 s (P to S in *DCMU* cells, M in $+$*DCMU* cells). Fluorescence excitation, $\lambda_e = 590$ nm, 300 μmol photons $m^{-2}$ $s^{-1}$, $\Delta\lambda = 20$ nm. See Fig. 4 for further details.

The presence of light induced state 2 to state 1 transition was also confirmed by 77 K fluorescence emission spectra in the species that showed the expected effect at room temperature (data not shown).

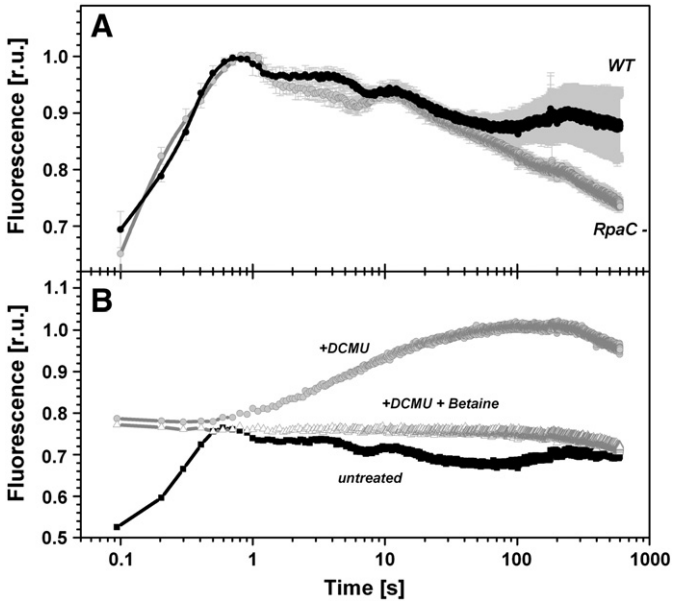


**Fig. 6.** Room temperature fluorescence induction measured at 682 nm (F682) with WT and RpaC$^-$ mutant of *Synechocystis* sp. PCC 6803. Panel A: comparison of WT and RpaC$^-$ induction of F682. Panel B: F682 induction of WT cells without any addition (*untreated*), with 10 μM DCMU (*DCMU*) and with 10 μM DCMU and 0.5 M glycine betaine (*+DCMU +Betaine*). Data were obtained from *spectrally resolved fluorescence induction* (SRFI — for further details, see Fig. 4) method with orange actinic excitation (590 nm, $\Delta\lambda = 20$ nm, 300 μmol photons $m^{-2}$ $s^{-1}$). Data in the Panel A represent average of 3 independent measurements; SD of particular points are marked by gray color.

The deconvoluted spectra were, however, used for calculations of PSII to PSI fluorescence ratios in selected species (Fig. 9). The high (or low) value of this ratio correlates with the presence of cells in state 1 (or state 2); therefore the relative increase in the ratio reflects state 2 to state 1 transition (see Fig. 9). The actinic irradiation caused increase of this ratio in untreated and DCMU treated cells of *Synechococcus* sp. (Fig. 9A —PCC 7942; Fig. 9B —WH 5701) and *Cyanothece* sp. ATCC 51142 (Fig. 9D). However, there was only a small increase in

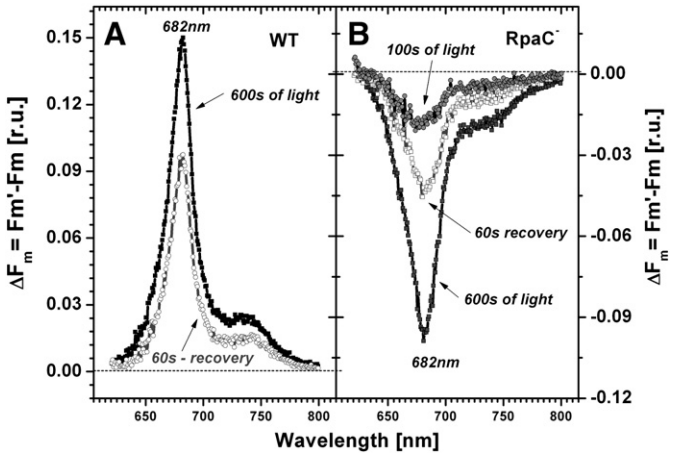


**Fig. 7.** Fluorescence difference spectra (illuminated minus dark) of maximal fluorescence for WT (A) and RpaC$^-$ mutant (B) of *Synechocystis* sp. PCC 6803. Data were calculated from numbers obtained from SRFI plots for wild-type cells (Fig. 4) and RpaC$^-$ mutant cells. ΔFm is the difference between $F_m'$ and $F_m$, where $F_m$ is the maximal fluorescence measured in dark and $F_m$' is the maximal fluorescence measured after a particular time of irradiation and after 60 s of recovery in dark (60 s recovery). Data represent average curves from n = 4.

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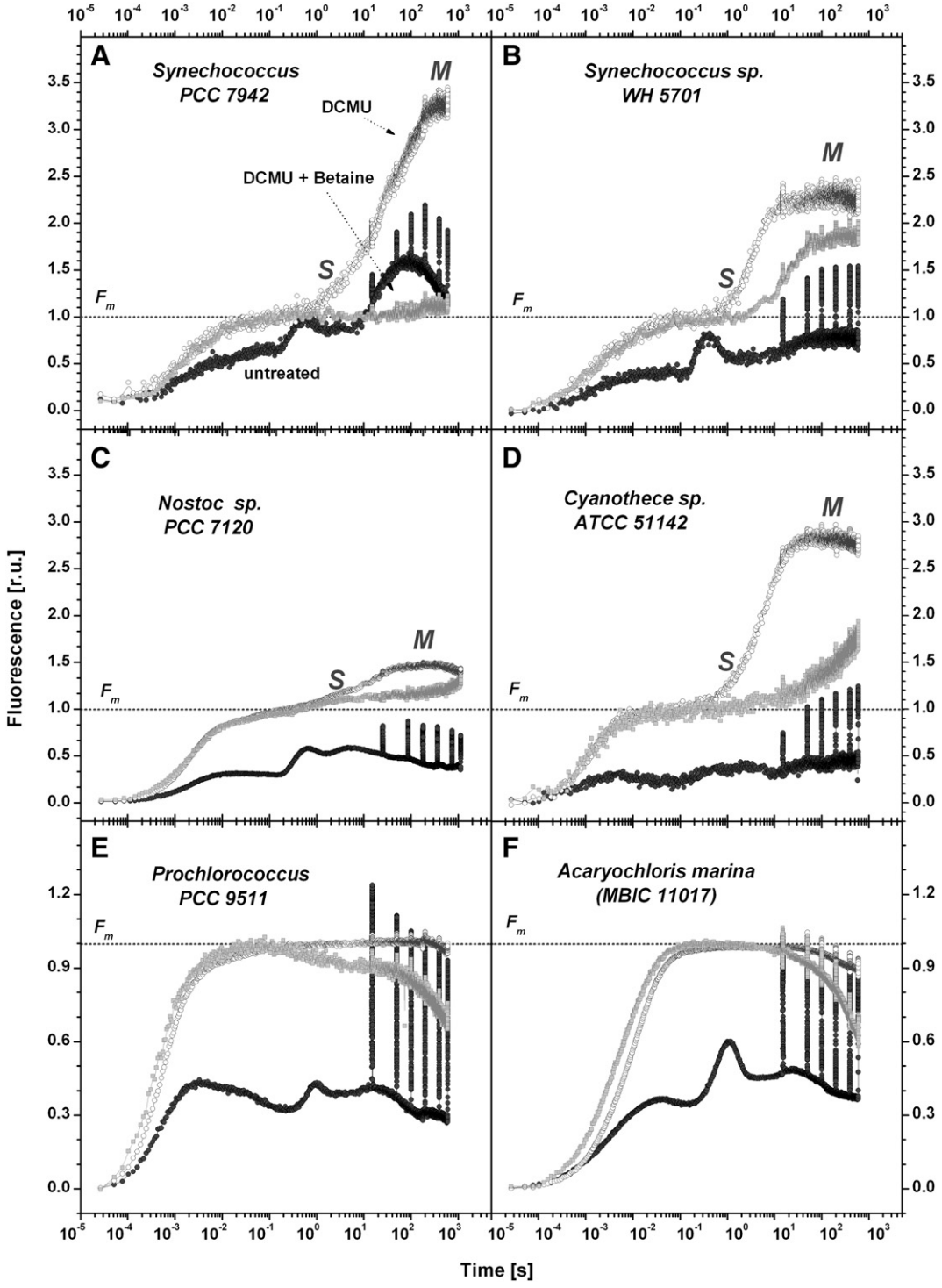


**Fig. 8.** Time course of chlorophyll *a* fluorescence changes measured with various cyanobacterial species as indicated. Samples without any addition (*untreated*; black line) or in the presence (as indicated) of DCMU (10 μM), nigericin (60 μM) and glycine betaine (*Betaine*, 500 mM). Six saturation light pulses were applied at 15 s, 50 s, 200 s, 430 s and 600 s to obtain $F_m'$. Data are normalized to the maximal fluorescence obtained after exciting samples with a saturating excitation pulse given before recording the fluorescence time course (see $F_m$ line). The S and M levels during the slow fluorescence transient are marked; see panel A for the key to different treatments for all other panels.

this ratio for *Nostoc* sp. PCC 7120 (Fig. 9C) in agreement with only a small increase in room temperature fluorescence induction in the presence of DCMU (Fig. 8C). This shows that light induced state 2 to state 1 transition, which we observed in high intensity orange light, proceeds in different degrees in the species used in this research (see Fig. 8). However, these data confirm that in all studied cyanobacteria containing PBS, the S to M fluorescence rise reflects the state 2 to 1 transition.

## 4. Discussion

In this paper we have provided experimental proof that, in PBS-containing cyanobacteria, the slow SM rise of Chl a fluorescence in the Fl kinetic trace is linked to the state 2 to state 1 transition. This was obtained, mainly, with WT *Synechocystis* sp. PCC 6803, and its RpaC$^-$ mutant that is locked in state 1 (Figs. 1 and 2); thus, the mutant is unable to undergo the state 2 to 1 transition [39,62]. In the

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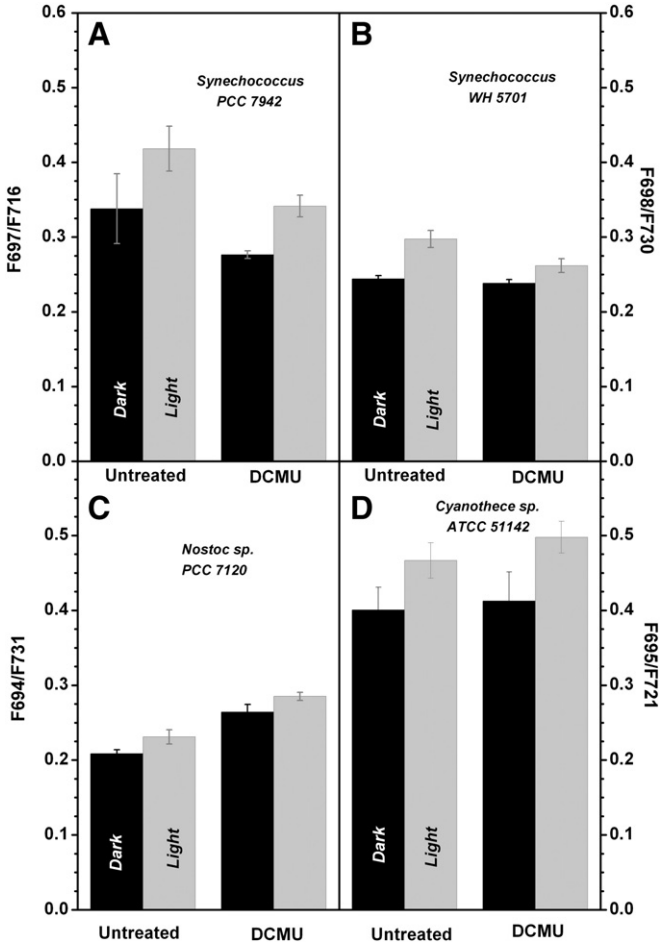


**Fig. 9.** The ratio of the 77 K fluorescence maxima of PS II/PS I measured with various phycobilisome-containing cyanobacteria. (A) *Synechococcus* PCC 7942 – here, fluorescence maximum of PSII was at 697 nm, and of PSI at 716 nm – thus, F697/F716 reflects fluorescence ratio of PS II/PS I; (B) *Synechococcus* WH 5701 – here, fluorescence maximum of PSII was at 698 nm, and that of PSI at 730 nm – thus, F698/F730 reflects fluorescence ratio of PS II/PS I; (C) *Nostoc* sp. PCC 7120 – here, fluorescence maximum of PSII was at 694 nm, and that of PSI at 731 nm – thus, F694/F731 reflects fluorescence ratio of PS II/PS I; (D) *Cyanothece* ATCC 51142 – here, fluorescence maximum of PSII was situated at 695 nm, and that of PSI at 721 nm) – thus, F695/F721 reflects fluorescence ratio of PS II/PS I. Standard deviation bars are for n = 3.

mutant, the SM rise is totally missing, as is also missing in hyperosmotic suspensions of WT cells (Fig. 3) which are locked in state 2 [54,63]. The ability of WT to perform a state 2 to 1 transition and the corresponding inability of the RpaC$^-$ mutant and of WT cells in hyperosmotic suspension to do the same, was also demonstrated by measuring 77 K fluorescence spectra of these cells and the F692/F726 band ratios (Figs. 1 and 2), as well as room temperature FI kinetics (Figs. 3 and 6).

Results, similar to those obtained with *Synechocystis* sp. PCC 6803 were obtained also with freshwater (PCC 7942; Fig. 8A) and marine (WH 5701; Fig. 8B) *Synechococcus* species, as well as with the filamentous diazotroph *Nostoc* sp. (Fig. 8C) and the single-celled diazotroph *Cyanothece* sp. (Fig. 8D). These cyanobacteria possess classical PBS, consisting of rods (assembled from PC or phycoerythrin hexamers) and cores (assembled from APC multimers), attached to the outer surface of thylakoids. The only cyanobacterial strain with classical PBS that did not show SM fluorescence rise when exposed to orange light was the thermophilic *Synechococcus bigranulatus* (data not shown), an extremophile and a close relative of *Thermosynechococcus elongatus*. In contrast, the SM rise is totally missing in *Acaryochloris marina* (Fig. 8F) that lacks PBS but has only phycobiliprotein rods, as well as in *Prochlorococcus* (PCC 9511; Fig. 8E) that lacks extrinsic phycobiliproteins altogether and has only intrinsic peripheral antenna pigments.

**Table 1**
Response of various cyanobacteria species to high intensity of blue and orange light irradiation. Effect of blue light (464 nm, 1000 μmol photons $m^{-2}$ $s^{-1}$) is characterized by parameter reflecting maximal fluorescence decrease — non-photochemical fluorescence quenching NPQ = ($F_m - F_m'$)/$F_m'$ (n.d. = not detectable) and by parameter of maximal fluorescence increase $\Delta F_m'$(rel) characterizing relative change in maximal fluorescence ($F_m'$) after irradiation — $\Delta F_m'(rel) = 100 * (F_m' - F_m)/F_m$. Effect of orange light (590 nm; 300 μmol photons $m^{-2}$ $s^{-1}$) is described by the presence or the absence of "SM rise" according to data shown in Figs. 3 and 8. Data represent average for n = 3. *S. bigranulatus* is an abbreviation for thermophilic *Synechococcus bigranulatus* (strain KOVROV 1972/8).

| | | **Synechococcus spp.** | | |
|---|---|---|---|---|
| | | *PCC* 7942 | *WH5701* | *S. bigranulatus* |
| Blue actinic light | NPQ<br>$\Delta F_m'$(rel) [%] | n.d<br>16.5 ± 2.5 | 0.194 ± 0.019<br>−16.2 ± 1.4 | 0.040 ± 0.043<br>−4.1 ± 3.4 |
| Orange actinic light | SM rise | Yes (high) | Yes (high) | No |
| | | **Synechocystis spp.** | | |
| | | *PCC 6803* | | |
| Blue actinic light | NPQ<br>$\Delta F_m'$(rel) [%] | 0.194 ± 0.035<br>−14.3 ± 2.3 | | |
| Orange actinic light | SM rise | Yes (small) | | |
| | | **Diazotrophic spp.** | | |
| | | ATCC51142 | PCC 7120 | |
| Blue actinic light | NPQ<br>$\Delta F_m'$(rel) [%] | 0.168 ± 0.038<br>−14.3 ± 2.8 | n.d.<br>1.9 ± 2.3 | |
| Orange actinic light | SM rise | Yes (high) | Yes (small) | |

The above results indicate that different mechanisms of state changes operate in cyanobacteria with varying architecture of light harvesting complexes. Currently, there exists no model for state transitions for those cyanobacteria that lack classical PBS. In *Acaryochloris marina*, there is a physical segregation of the PSI and PSII complexes [57] that suggests a minimal role of the excitation spillover mechanism for balancing the distribution of electronic excitation between the two photosystems. Moreover, since its phybiliproteins are almost immobile [64], the question arises whether a balanced excitation distribution can be achieved by means of state transitions. However, since *Acaryochloris* does not display the SM rise of Chl *a* fluorescence (Fig. 8F), the involvement of a regulatory state transitions mechanism is questionable.

Even less is known about state transitions in *Prochlorococcus* that contains only membrane-bound Chl *a*/*b* antennae (PCB antennae) and no functional phycobiliproteins [58,65]. However, state transition was described in *Prochlorothrix hollandica* [66], also a PCB binding prochlorophyte [65] that involves phosphorylation of PCB proteins [67]. This mechanism is similar to that of plants and algae that involves phosphorylation of intrinsic Chl–protein complexes [67] than of the PBS-containing cyanobacteria.

In cyanobacteria with classical PBS, the state transitions are usually interpreted as an adaptive mechanism that balances the turnover rates of the two photosystems under low, non-saturating light intensities [8]. State transitions in these cyanobacteria may be the result of translational or orientational movement of PBS from a position that favors excitation energy transfer to PS II Chls *a* (state 1) to a position that favors excitation energy transfer to PS I Chls *a* (state 2; [17,23]). According to [39,62], the RpaC protein is part of the PBS–PSII complex and acts by maintaining an optimal binding of the PBS to PSII, *i.e.* neither too tight nor too unstable. Our results with hyperosmotic cell suspension and with the addition of uncoupler nigericin support this interpretation. WT cells in state 1 are driven to state 2 either upon hyper-osmosis in few milliseconds [53] or by collapsing the transmembrane ΔpH with protonophores like nigericin. Both treatments, however, failed to drive the state 1-locked RpaC$^-$ cells to state 2 (Figs. 1 and 3). Therefore, in the absence of the RpaC protein, PBS binds to PSII quite strongly. RpaC

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deficiency also influences the fluorescence response — instead of a fluorescence SM rise, we have detected a small quenching in maximal fluorescence ($F_m$) with its maximum at 682 nm (see Fig. 7).

We have also shown that the capacity for state transitions in cyanobacteria (as inferred from the SM fluorescence increase) varies among different species (Figs. 8 and 9 and Table 1). It is most dominant in *Synechococcus* (especially in the PCC 7942 strain), the common model organism for SM rise studies [13,47,68,69] — where the $F_t$ value can increase well above the $F_m$ value (Fig. 8A). In contrast, SM phase is rather small in *Synechocystis PCC 6803* (Fig. 3) and in *Nostoc* sp. (Fig. 8C). This suggests that *Synechococcus* PCC 7942 and WH 5701 cope with the sudden increase of irradiance in a different manner than *Synechocystis* (*e.g.* PCC 6803). In *Synechococcus* sp. PCC 7942 overexcitation by orange light stimulates state transition changes to higher extent than in *Synechocystis* sp. (PCC 6803). This is also reflected in the larger light-induced increase in $F_{PSII}/F_{PSI}$ emission ratios at 77 K (~30% changes in Fig. 9A in the case of *Synechococcus* compared to only 10% change in Fig. 2A) and also in the room temperature fluorescence induction (compare Fig. 8A with Fig. 3A in the case of *Synechocystis*). There is only 50% fluorescence increase during S to M induction in *Synechocystis* PCC 6803 (Fig. 3A) but almost 200% increase in *Synechococcus* PCC 7942.

Interestingly, *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 are also different in their reaction to blue light (compare *e.g.* [47] and [70] and Table 1). While blue light induces fluorescence quenching due to the action of the Orange Carotenoid Protein (OCP) that is present in most of *Synechocystis* sp., including PCC 6803 [51,52], but in Synechococcus PCC 7942 blue light induces rather fluorescence rise [47] as OCP protein is missing in this strain (for details see [70]). To explore the relation between the SM fluorescence rise in orange light and the quenching of fluorescence in blue light for more cyanobacteria, we measured in all the studied strains blue light-induced NPQ together with the parameter $\Delta F_m'(rel)$ that represents relative changes in maximal fluorescence after irradiation — $\Delta F_m'(rel) > 0$ indicates the presence of fluorescence increase (SM rise), $\Delta F_m'(rel) < 0$ shows the presence of decrease in maximal fluorescence because of fluorescence quenching (see NPQ in Table 1). Table 1 shows that in cyanobacteria strains supposedly without OCP [70] (*e.g.* PCC 7942 or *Synechococcus bigranulatus*, a close relative of *Thermosynechococcus elongatus*) the NPQ is either totally missing (PCC 7942) or negligible (*Synechococcus bigranulatus*). The parameter $\Delta F_m'(rel)$ is a different expression of fluorescence quenching (de-quenching) similar to the NPQ. However $\Delta F_m'(rel)$ allows a quantitative comparison of the extent of NPQ (fluorescence quenching) and SM rise (fluorescence de-quenching) in equivalent values, it means as a relative change in maximal fluorescence in light (decrease for NPQ or increase for SM rise). In a case of $\Delta F_m'(rel) > 0$ the parameters show the relative extent of energy diverted from photochemistry to fluorescence increase – SM rise; for $\Delta F_m'(rel) < 0$ the parameter shows the extent of energy diverted from photochemistry into heat by non-photochemical fluorescence quenching — NPQ. For example, in the strains with OCP dependent blue-light induced non-photochemical quenching (*Cyanothece* sp. ATCC 51142; *Synechococcus* sp. WH 5701; *Synechocystis* sp. PCC 6803) the observed NPQ can be recalculated in terms of $\Delta F_m'(rel)$ to be around −16% (see Table 1) that shows relative fluorescence decrease by 16% due to NPQ action. Interestingly this is very close to the extent of fluorescence SM rise in PCC7942 (17%, see Table 1) that does not have OCP protein and thus cannot induce NPQ [70]. Therefore, we suggest that the maximal fluorescence increase (see $\Delta F_m'(rel)$ in Table 1) during the SM rise may represent light-induced energetic decoupling of PBS from photosystems that acts as a photoprotective mechanism for safe dissipation of excessive irradiation into fluorescence. Indeed, such a pronounced increase in PBS fluorescence can be detected *in vivo* in PCC 7942 [47]. Our results are also in agreement with the recently suggested role of phycobilisome detachment during high-light stress in cyanobacterium *Synechocystis* sp. PCC 6803 [71] and phycobilisomes decoupling observed in diazotrophic cyanobacterium *Trichodesmium* [72].

In this paper, we have shown that phycobilisome decoupling seems to be important not only during strong irradiation (see *e.g.* [71]) but also during lower, physiological irradiance (300 μmol photons $m^{-2}\,s^{-1}$). The protective role of phycobilisome decoupling has already been suggested for red algae [73] but not for phycobiliproteins containing cryptophytes that are protected by very efficient NPQ in their chlorophyll a/c antennae [48]. We suggest that phycobilisome decoupling from photosystem II during state transitions is important not only for the equilibration of light absorption between photosystems at low light but that it also plays an important photoprotective role at high light. This is in agreement with the observation that RpaC (a protein essential for PBS decoupling from photosystem II [39]) is essential for viability of *Synechococcus* PCC 7942 (cyanobacterium without blue light induced NPQ [70]) but not for *Synechocystis* PCC 6803 [62] with NPQ (Table 1). In cyanobacterial species like *Synechococcus* PCC 7942 that lack OCP induced NPQ [70], fluorescence increases after actinic light irradiation could represent a safety valve against overexcitation. However, the existence of two mechanisms (*i.e.* OCP controlled NPQ in blue actinic light, and orange light-induced SM fluorescence rise) in one organism does not seem to exclude one from the other (see Table 1). For instance, in *Cyanothece* sp. and in *Synechococcus* WH 5701 the presence of pronounced SM rise on orange light excitation (Fig. 8D) is accompanied also by blue light-induced quenching (Table 1). Therefore, a detailed understanding of mechanisms triggering SM fluorescence rise (*e.g.* its inhibition by anaerobiosis [74]), its interconnection with OCP dependent NPQ, and physiological importance of SM fluorescence rise must be resolved as cyanobacteria without OCP are more sensitive to short periods of excessive irradiation [70].

*In conclusion*, results presented in this paper prove that the pronounced SM fluorescence rise is due to state 2 to state 1 transition. It is present in various cyanobacterial species that possess complete PBS. We suggest that SM fluorescence rise represents an alternate mechanism for the dissipation of excess excitation energy in cyanobacteria that lack other photoprotective mechanisms.

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