LIGHT ACCLIMATION OF UNICELLULAR RED ALGA *RHODELLA VIOLACEA*: ΔpH BUILD UP, ANTENNA REASSEMBLING AND PHOTOPROTECTION

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Abstract. Thylakoid membranes of red algae contain water-soluble membrane-bound complexes – phycobilisomes (PBSs) serving as peripheral antennae for photosystem II (PS II). Strong light absorbed by the PBSs triggers a fast formation of transthylakoid ΔpH that follows the non-photochemical quenching of chlorophyll (Chl) fluorescence. The ΔpH build-up seems to be essential for photoprotecting the photosynthetic apparatus in the absence of xantophyll cycle common to higher plants. However, the mechanisms of this process are yet to be studied in detail. Here we report on study the Chl fluorescence quenching in unicellular red algae *Rhodella violacea* and its correlation with the ΔpH gradient being formed. The relation of this phenomenon to PS II photoprotection in the normal and high light-acclimated Rhodella cells is also examined. Under the photoinhibitory conditions (white light of 2000-3000 μE/m2s), the ΔpH -dependent Chl fluorescence quenching was found to delay the kinetics of PS II photoinhibition. The uncouplers like nigericin and NH_4Cl are known to break down ΔpH gradient and lead to the dissipation of Chl fluorescence quenching followed by enhancing the PS II photoinhibition rate. The same effect showed far-red (FR) light transthylakoid ΔpH consumption. ATPase inhibitor (DCCD) having no impact on ΔpH didn't influence PS 2 photoinhibition as well This implies the photoprotection to be fulfilled by the proton gradient rather than by ATP synthesis. Light acclimation of Rhodella cells to higher irradiances (500-1000 μE/m^2s) results in a partial loss of the periphery phycoerhytrin-containing subunits by PBSs. Here we show that the light-acclimated cultures display a higher resistance to the photoinhibitory light than the non-acclimated ones. This could be explained by diminishing the energy transfer from the reduced PBSs to PS II and light screening by the secondary carotenoids synthesized during light exposure. Low-temperature (77K) fluorescence data allowed to evaluate the molecular mechanisms contributing to suppressing Chl fluorescence in Rhodella cells and its recovery in darkness.
Key words: *antenna reassembling, photoprotection, Rhodella violacea.*
Abbreviations: LL, low light (40 μE/m^2s); HL, high light (500 μE/m^2s); XHL, extra-high light (1000 μE/m^2s); Chl, chlorophyll "a"; PBS, phycobilisome(s), PE, B-phycoerythrin; PS 1&2, photosystem 1&2; PI, photoinhibtion; ETC, electron transport chain

INTRODUCTION

All phototrophic organisms are capable of photosynthesis, i.e. absorption and transformation of solar energy resulting in synthesis of reduced compounds. Higher plants and algae produce molecular oxygen, a by-product of photosynthetic electron transport. The primary photosynthetic reactions are performed by pigment-protein complexes (CPs) embedded in thylakoid membranes [20].

The photosynthetic CPs comprise the reaction centers of photosystem 1 (PS 1), those of photosystem 2 (PS 2), and light-harvesting antenna complexes (LHC). Red algae and cyanobacteria differ from higher plants by lacking of large distant antenna complex LHC II. Instead of it, they have extramembrane antenna complexes, phycobilisomes (PBSs), attached mainly to PS 2 from the stromal part of thylakoids [13].

While photon flow is required for proper functioning of the photosynthetic electron transport chain (ETC), the excessive light intensity can inhibit photosynthesis and inflict irreversible damage to pigments and proteins of thylakoid membranes [1, 3].

Plants developed various photoprotective mechanisms to resist light stress [6, 11, 23, 24]. However, if these mechanisms of excessive energy relaxation are insufficient, the remaining flux of photons leads to the photosensitized formation of triplet chlorophyll and toxic singlet oxygen. The first target of photoinhibition is the PSII reaction center, which is associated with a loss of photochemical activity and variable fluorescence Fv [2, 7, 17, 19, 27, 28, 30].

One of the photoprotective mechanisms described for higher plants involves a down-regulation of PS 2 that proceeds simultaneously with the build-up of a proton gradient across the thylakoid membrane (ΔpH) [11]. It is often correlated with deepoxidation of xanthophylls located in LHC2 [6, 11, 14, 25, 29].

In *Rhodophyta* peripheral antennae – PBSs transfer excitation to core antennae of PS2, CP43 and CP47 protein-chlorophyll complexes where xanthophyll cycle is absent [13, 21]. However, a ΔpH-dependent Chl a fluorescence quenching can also be formed under strong light [8, 18]. Possible photoprotective role of ΔpH was studied for unicellular red alga *Rhodella violacea* [21, 22]. As a long-term response to light stress, photoacclimation takes place. This requires photoregulation of gene expression and several changes at the cellular level [22].

Studies on photoacclimation have compared the steady-state exposures to high light (HL) with the low light (LL) exposures. These investigations focused on various aspects of the differences between acclimated and LL-grown cells at the level of their ultrastructure, pigment content, light harvesting antenna size and PSII to PSI ratios [8, 26, 22].

The aim of the present research was to study different photoprotective mechanisms in red alga *Rhodella violacea*. In response to high light, the cells of Rhodella trigger a fast generation of transthylakoid ΔpH accompanying the non-photochemical quenching of Chl *a* fluorescence [8, 18]. We characterised this quenching, studied the effects of various irradiances and inhibitors. Under photoinhibitory conditions, the ΔpH-dependent Chl fluorescence quenching exerts a photoprotective role and delays the kinetics of photoinhibition [21, 22]. Here we provide additional evidence for photoprotective role of ΔpH-mediated non-photochemical Chl fluorescence quenching (NPQ) formation in LL-grown Rhodella cells and strong light acclimated ones as well.

Long-term acclimation of Rhodella to high light is accompanied by the reduction of antenna size (loss of the distal hexamers containing B-phycoerhitrin (PE) [4]. We investigated the acclimation and its contribution to photostability of photosynthetic machinery of Rhodella.

MATERIALS AND METHODS

Rhodella violacea (strain 115-79 from Goettingen University, Germany) was grown photoautotrophically in sterile artificial seawater with the addition of vitamin B12 at 25 mg L21 [8, 9]. Cultures of 300 to 700 mL were incubated at 20°C in glass culture flasks continuously flushed with sterile air, and illuminated with fluorescent tubes with a 16-h light/8-h dark photoperiod [21, 22].

Cultures of 3-4 day-old were used for the experiments. Three different light intensities were used for the light acclimation: 40, 500, and 1000 μE/m^{2}s, defined as LL (low light), HL (high light), and XHL (extra high light) conditions. To standardize the culture conditions and minimize self-shading, cells were diluted every 3 days to 700 cells /ml with fresh medium. Full adaptation took 7 days; the cultures were diluted to 700 cells/ml 3 days prior to the photoinhibition experiments.

Rhodella suspensions were photoinhibited in a thermostated vessel (20ºC) by white light at photon flux densities 2000-4000 μE/m^{2}s. Photoinhibited samples were stored in the dark at 20° C for 15 min prior to fluorescence induction measurements to provide full dissipation of NPQ [4, 21, 22].

Fluorescence induction kinetics of the PSII Chl at 20°C were measured in a laboratory-built continuous fluorimeter using green LEDs (λ =550 +/-20 nm, I=40 μE/m^{2}s) to provide continuous illumination of adjustable intensity and duration that served both as actinic and as detecting beam. The fluorescence was detected at 680 nm by a photomultiplier H 5700–50 (Hamamatsu Photonics, Japan). Data were collected in a computer with a 33-μs time resolution. The results were displayed on logarithmic time scale to present all phases of the kinetic curves together [21, 22].

At room temperature, fluorescence emanates mainly from the Chl a antenna of PSII [16]. Fluorescence yield is dependent on photochemical quenching and NPQ [26]. Photochemical quenching is dependent on the redox state of the primary acceptor of PSII, the plastoquinone Qa [16, 20]. When all PSII centers are open (Qa oxidized), they are efficient exciton traps and the fluorescence yield of PSII is low. When Qa is reduced, the centers are unable to trap excitons, the photochemical quenching is suppressed. Chl fluorescence can also be decreased by NPQ ascribed to two main processes occurring under strong light: ΔpH-dependent quenching and photoinhibition [8, 9, 18, 21-23, 26]. In the absence of photochemical quenching and NPQ, fluorescence reaches a maximum level Fm. The ΔpH-dependent quenching can be destroyed by nigericin (an uncoupler functioning as a proton-transporting ionophore) [9, 21, 22].

Low-temperature fluorescence of Chl and other photosynthetic pigments was detected in liquid nitrogen (77K) in Hitachi Spectrofluorimeter (Hitachi, Japan). Fluorescence band ratio PS 2/PS 1 was calculated to characterize any photodamage or changes of photosystems in the result of photoregulatory processes. Both excitation and emission spectra of 77 K fluorescence were used to estimate energy migration efficiency from PBSs to PS 2 and PS 1.

Absorption spectra of Rodella suspension were registered at 20°C in double-beam Aminco spectrophotometer equipped with Shibata plate to diminish light scattering.

RESULTS

Photoprotective role of ΔpH-mediated non-photochemical Chl fluorescence quenching.

Similar to other photosynthetic organisms, dark-adapted Rhodella cells demonstrate typical OIDPS fluorescence induction curves under continuous irradiation (green light, I=40 μE/m^{2}s) (Fig. 1). The initial value of Fo corresponds to fluorescence of Chl in photosynthetic antennae when most of PS 2 reaction centers (RC) are open [18]. Once light photons are absorbed, primary and secondary quinone electron acceptors of PS 2 (Qa and Qb, correspondingly) are reduced and fluorescence reaches Fi maximum; this process takes about 20 microseconds. Since the double-reduced Qb migrate to plastoquinone pool (PQ) with slower rates compared to primary reactions, the Fi peak corresponds to the fully reduced Qb. Fluorescence reaches Fp maximum when all plastoquinones are reduced; usually 500-1000 ms is needed to that. Finally, the long-term monotonous decline of fluorescence is observed. This loss of fluorescence is considered to originate from non-photochemical quenching following the transthylakoid ΔpH build up. After the actinic light was turned on NPQ developed for 80-100 sec.

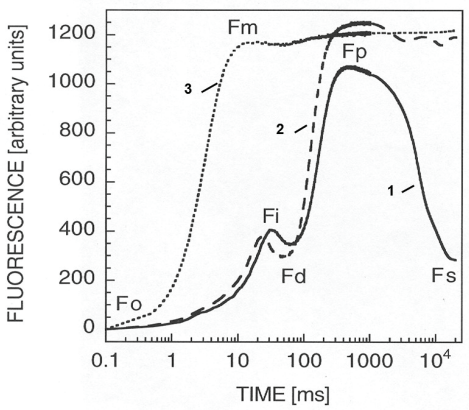

Figure 1. Fluorescence induction curves of LL-grown Rhodella cultures. Continuous fluorimeter with green LED excitation. Chl concentration - 5 μg/ml. (1) dark adapted cells grown at LL; (2) cells preincubated with 80 μM of nigericin; (3) cells preincubated with 10 μM of DCMU

Since PQ pool is always in an unstable equilibrium (strongly dependent on light intensity and spectrum) between Qb and Cyt b6f-complex consuming electrons from PQ to reduce PS 1, Fp amplitude is lower than the real maximal fluorescence [21, 26]. To obtain the maximal value Fm researchers usually add DCMU, which blocks the electron transport between Qa and Qb preventing ΔpH formation [18, 21]. When we loaded 10 μM DCMU to Rhodella cells prior to light irradiation we observed the fluorescence rise to Fm in 10 μsec (Fig. 1). To obtain solid evidence on the correlation between the ΔpH gradient and NPQ we added nigericin, a proton transporting ionophore disrupting transthylakoid ΔpH. The induction curve retains its shape reaching Fm level, but without Fs dip that reflects likely total dissipation of a non-photochemical quenching.

There are several findings pointing at ΔpH buil up to be a fast photoprotective mechanism when light induced ΔpH-mediated protein conformational changes in antennae result in enhance of thermal dissipation of excess energy.

According to this suggestion, nigericin added to Rhodella suspensions in concentration of 80-100 μM enhanced photoinhibition rates, as it was measured by kinetics of Fv loss (variable Chl fluorescence, Fv= Fm (Fp) – Fo). Adding another uncoupler, NH_4Cl demonstrated the same effect although the effective concentrations were 10 times exceeding those for nigericin and, according to the fluorescence induction curve, a part of NPQ remains (data not shown).

In full agreement to the above statement, DCCD, inhibitor of chloroplast ATPase which has no effect on ΔpH gradient, didn't change kinetics of PS 2 photoinhibition [9].

One more evidence of a photoprotective role of transthylakoid ΔpH was found when far red irradiation was applied. Far red light (FR, $710<\lambda<760$ nm) absorbed mainly by PS 1 brings about the dissipation of ΔpH and restoration of the Chl fluorescence (that is, dissipation of ΔpH-induced Chl fluorescence quenching). This effect was attributed to activating the chloroplast ATPase via a thioredoxine system [9, 21]. In the extra set of experiments we examined whether FR influences the photoinhibition rates.

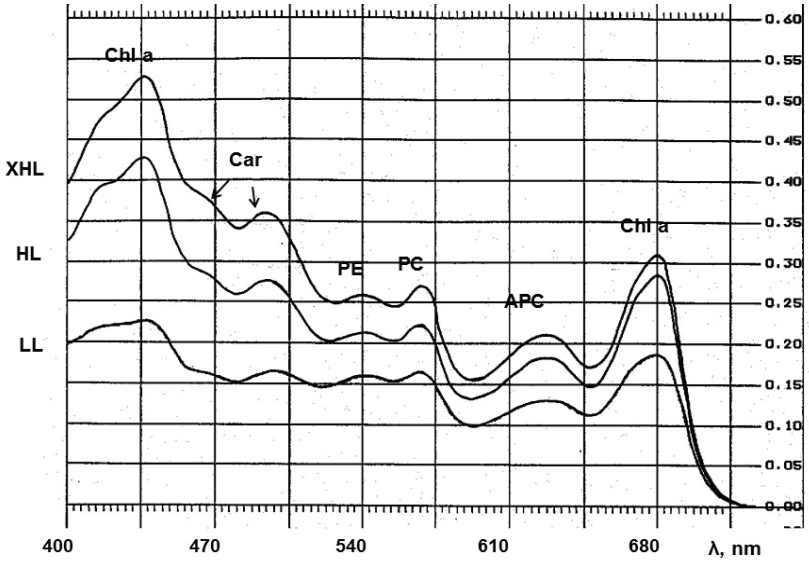

Figure 2. Absorption spectra of LL-grown and light-adapted Rhodella suspensions at 20^0C. Chl concentration – 5 μg/ml. Optical pathway ***l*** = 1 sm. LL – low light – 40 μE/м^{2}с. HL – high light – 500 μE/м^{2}с. XHL – extra high light – 1000 μE/м^{2}с. PE – B-phycoerithrin, PC – C-phycocyanin, APC – allophycocyanin

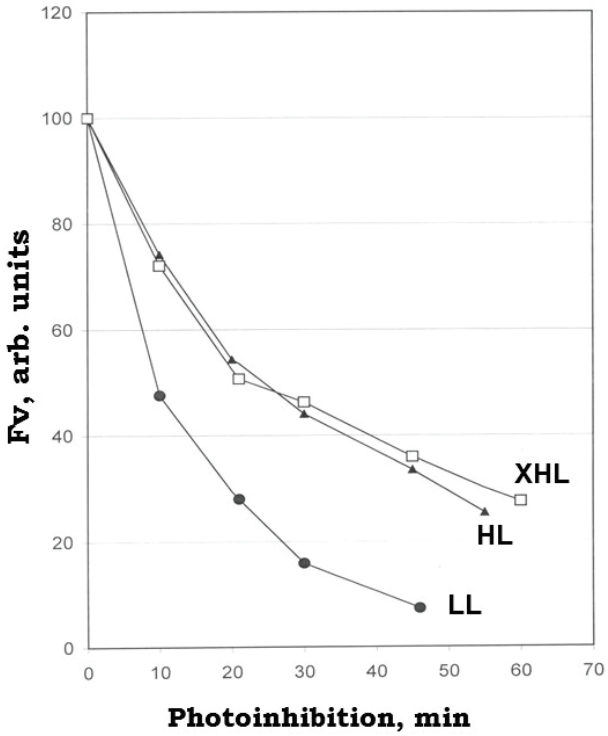

Figure 3. Kinetics of PS 2 photoinhibition in low light-grown and high ligh-acclimated Rhodella cells. Photoinhibition: white light (halogen lamp, I= 4000 μE/м^{2}с). [Chl] = 5 μг/мл, $t^0 = 20^0$C. LL – 40 μE/м^{2}с. HL – 500 μE/м^{2}с. XHL – 1000 μE/м^{2}с

Simultaneous exposure of Rhodella suspensions to white light and FR slightly affected kinetics of photoinhibition obtained in the absence of FR. It is reasonable since FR source provided much less light intensity and was unable to dissipate ΔpH being formed under white light.

However, when we applied short (2 min) FR and white light expositions alternately, that is maintained an average lower level of ΔpH, we have observed a clear enhancement of photoinhibition rates even in LL grown Rhodella cells; the difference was about 25% after 20 min of light exposure (data not shown). These results confirm the importance of the fast transthylakoid ΔpH build up to resist the algal photosynthetic apparatus against strong light.

Acclimation to strong light of LL-adapted Rhodella cells.

The low light grown Rhodella cultures were acclimated to strong light from 3 to 7 days. LL-grown cultures were transferred to HL or XHL intensities. Such adaptation was previously shown to cause partial loss of PE-containing distal hexamers in PBS antenna and suppression of synthesis of the PE chromophore [22]. This phenomenon can be visualized as a progressive change in the colour of the cultures, which turn greenish.

As it follows from Figure 2, despite of equal Chl content (5 μg Chl/ ml) in all three samples (LL, HL, XHL) their absorption spectra differ from each other. Changes in the blue part of absorption spectra of the Rhodella suspensions indicate the lower content of PE and increased carotenoid bands. The excessive amounts of carotenoids are known to be accumulated in response to light stress and the effect is common for numerous species of microalgae. These "secondary" carotenoids localize in lipid particles outside photosynthetic membranes so that they probably serve as a "screen" to protect chlorophyll proteins from the strong light [5, 25].

Because of a more friable thylakoid packing in the light-adapted cultures, the seeve effect was different among LL, HL and XHL samples resulting in the different OD value in the red Chl absorption peak. That is why we measured the total light absorption (1-T) by these suspensions and took these values for final correction of the photoinhibitoin curves.

Light adaptation and photoinhibition.

Since photoinhibition of PS 2 is a complex physiological process induced by oversaturation of electron transport via PS 2 ("acceptor-side photoinhibition") the influence of photodamage rates on the effective antenna size seems to be natural [3].

We have studied PS 2 photoinhibition in Rhodella cultures adapted for strong (Hl and XHL) light. Results show that at 2000-4000 μE/m^{2}s photon fluxes the photoinhibition kinetics were: LL>HL>XHL (Fig. 3). The difference between HL and XHL was clearly less than one between HL (XHL) to LL probably because of a complete detachment of PE hexamers even under HL light intensity (500 μE/m^{2}s).

The obtained data demonstrate the kinetics of Fv loss to be delayed in HL and XHL-adapted culters as compared to the LL-grown ones. This is consistent with the fact that PBSs in Rhodella are preferentially attached to PS 2 rather than to PS 1, so a smaller antenna size in high light-grown cells prevents PS 2 from excessive energy flow and decreases the photodamage.

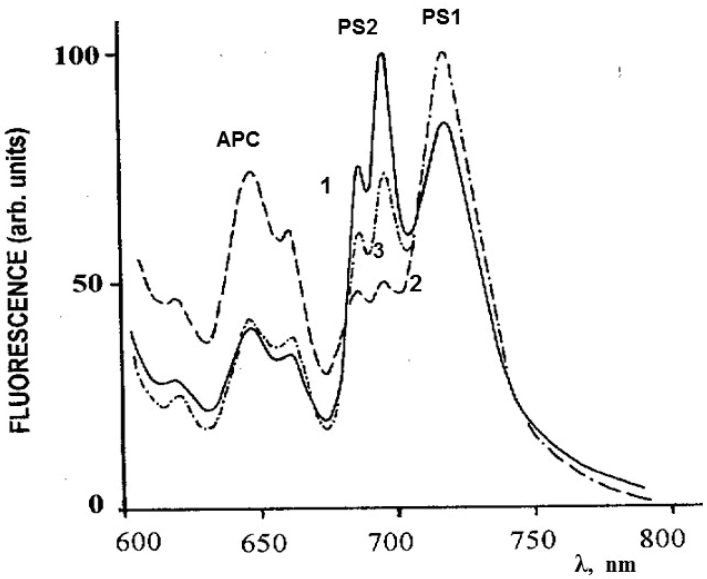

Figure 4. Low temperature (77K) fluorescence of pigments in the cells of *Rhodella violacea.* 1 – control cells (LL-grown). 2 – Photoinhibition: 25 min. 3 – Photoinhibition: 25 min + dark recovery 2 h

Interestingly, FR irradiation enhanced the photoinhibition rates in all three types of Rhodella cultures what confirms the above suggestion that ΔpH gradient accompanied by NPQ is the fastest and possibly the most universal photoprotective mechanism existing in red algae.

Low temperature fluorescence and "state transitions-like" mechanism.

Fluorescence spectra of thylakoid membranes at 77K provide valuable information on as PS 2 as PS 1 since Chl fluorescence at the liquid nitrogen temperature is emitted by both photosystems. Under the excitation at 560 nm (PE absorption maximum in phycobililisome) we detected in Rhodella cells fluorescence of PS 2 at 686 and 696 nm and of PS 1 at 720 nm. Additional characteristic maximum at about 645 nm was observed and prescribed to AFC, the terminal energy acceptor in the core part of phycobilisomes. In contrast to PE protein complexes APC core proteins were stable during the light adaptation to high irradiances, so that this band was used as an amplitude marker.

We have shown that the photoinhibited Rhodella cells manifest a decrease of both PS 2 and PS 1 fluorescence bands referred to APC emission (Fig. 4), likely, because of the photodamage done to both photosystems with their integrity being impaired. Dark recovery of the photoinhibited cultures demonstrated a partial restoration of PS 2 and PS 1 bands and increase of PS 2 / PS 1 ratio. Faster restoring of PS 2 may be explained by a *de novo* synthesis and a subsequent turnover of D1 protein; the process is light inducible [28].

Since the 77K fluorescence was excited by green light absorbed mainly by PE we have calculated the ratio of Chl and PE maxima in fluorescence excitation spectra during high light adaptation of Rhodella cultures. We found that during 72 hours of adaptation of LL-grown cultures to HL ratio of emission bands F686/F648 (PS2 band to APC band) declined and F718/F648 (PC1 band to APC band) increased. The ratio of excitation maxima E572/E681 (PE band to Chl band, fluorescence detection at 735nm) increased supporting the ideas that part of PBSs detaches from PS 2 and move to PS 1 increasing energy migration to PS 1. This process resembles the "state transition", a light adaptation mechanism descried for higher plants although in this case it occurs within a longer time scale [8]. Alternatively, the described effect may be explained in terms of light-accelerated *de novo* PBS synthesis where the newly synthesized phycobilisoms associate preferentially with PS 1 [4]. Clearly, this phenomenon requires further investigations.

CONCLUSION

The fastest mechanism of photoprotection in red algae is the light-induced build up of transthylakoid ΔpH, which may be easily followed by the non-photochemical fluorescence quenching [8, 21]. The important role of ΔpH-gradient in photoprotection was confirmed by the photoinhibition acceleration under FR light known to reoxidize the plastoquinone pool and enhance the dark dissipation of ΔpH.

The obtained results reveal that the acclimation of *Rhodella violacea* to strong light (500 or 1000 μE/m^{2}s) leads to a better photostability of its photosynthetic machinery. The reasonable explanation may run as follows: when the antenna size is reduced (detachment of PE-containing distal proteins), the energy flow to PS 2 discharging exciton pressure to ETC also goes down.

This subsequently suppresses the photoinhibition rates in HL and XHL acclimated cells. It is worth mentioning that photoinhibition kinetic curves for HL and XHL samples were close to each other pointing at the possibility that both PE hexamers in PBS rods to detach under HL irradiance, hence, the increase of light intensity to XHL didn't additionally reduce the antenna size.

"Non-photosynthetic" secondary carotenoids, synthesized under high irradiance and accumulated outside the photosynthetic membranes can provide additional barrier against the photon excess.

According to the data on 77K fluorescence, the photoinhibitory light is destructive for both PS 2 and PS 1 though the photosystems are capable to recover partially in darkness. PS 2 recovers faster than PS 1 obviously because fast renewability of the key D1 protein.

Acclimating the Rhodella cells to high light for 72 h results in a decrease of energy transfer from PBS to PS 2 and simultaneous increase of PBS-to-PS 1 energy migration. These preliminary results obtained by analyzing both the emission and excitation fluorescence spectra point to the "state-transitions-like" process going on the light-acclimating Rhodella cells. Although the state transitions such as an adaptive mechanism had been described exclusively for higher plants, they could also exist in red algae). However, this issue has to be further elucidated.

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