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D1 protein turnover is involved in protection of Photosystem II against UV-B induced damage in the cyanobacterium *Arthrospira* (*Spirulina*) *platensis*

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ABSTRACT

By using two strains of *Arthrospira (Spirulina) platensis*, an economically important filamentous cyanobacterium, we compared the impairment of PSII activity and loss of D1 protein content under UV-B radiation. Our study showed that UV-B radiation induced a gradual loss of the oxygen-evolving activity to about 56% after 180 min UV-B irradiation both in strains 439 and D-0083, which have been kept under indoor and an outdoor culturing conditions, respectively for a prolonged period of time. The loss of oxygen evolution was accelerated in both strains in the presence of lincomycin, an inhibitor of protein synthesis, and the amount of D1 protein showed a decrease comparable to that of oxygen evolution during the UV-B exposure. However, the UV-B induced loss of oxygen-evolving activity and D1 protein amount was largely prevented when *A. platensis* cells were exposed to UV-B irradiance supplemented with visible light. Comparison of the two strains also showed a smaller extent of D1 protein synthesis dependent PSII repair in the indoor strain. Our results show that turnover of the D1 protein is an important defense mechanism to counteract the UV-B induced damage of PSII in *A. platensis*, and also that visible light plays an important role in maintaining the function of PSII under simultaneous exposure to UV-B and visible light.

1. Introduction

Enhanced solar UV-B radiation reaching the Earth's surface due to thinning of the stratospheric ozone layer has received considerable attention in recent years because of its potential adverse effects on the biosphere, including photosynthetic organisms [1]. Cyanobacteria, represents the largest and most widely distributed group of photosynthetic prokaryotes on the Earth. They have been regarded as useful model organisms for studying the UV-B damage and repair mechanisms because they were exposed early in their evolution to UV-B radiation that was much higher than the present day level [2]. It has been reported that UV-B radiation could alter the morphology [3], impair the motility and photoorientation [4], and inhibit the growth, nitrogen metabolism [5] and photosynthesis [6–8] of cyanobacteria. However, cyanobacteria living in their natural habitats possess different defense strategies counteracting the harmful effects of UV-B radiation, such as behavioral avoidance [9], production of UV-absorbing compounds [10], photoreactivation and excision repair of UV-B damaged DNA [11], and *de novo* synthesis of the D1 and D2 proteins for the damaged Photosystem II complex [12,13]. Recently studies have shown that in order to cope with UV-B induced photoinhibition of PSII, cyanobacteria can regulate multiple *psbA* genes to boost the expression of D1 protein, or to exchange the constitutive D1:1 protein to an alternate D1:2 isoform [14]. The ability of cyanobacteria to avoid or repair UV-B induced damages determines their capability to tolerate the adverse effects of UV-B radiation.

Arthrospira (*Spirulina*) *platensis* is a filamentous cyanobacterium capable of growing in extreme alkaline habitats. It has long been cultured for production of human health food [15] and animal feed [16] due to its valuable constituents. Therefore, considerable efforts have been devoted to optimize its growth conditions for massive production [17–19]. Recently, negative impacts of UV-B and positive effects of UV-A on its growth have been reported [20,21]. UV-B is known to harm the anchor polypeptide (85.5 kDa) and Chl antenna complexes, decrease the photochemical efficiency, inhibit O_2 evolution, damage DNA and alter the spiral structure of *A. platensis* [3,20,22–25]. In contrast to many other cyanobacteria, however, *A. platensis* does not accumulate UV-absorbing compounds to counteract the deleterious UV radiation. The enhanced self-shading associated with the UV-induced compression of the spirals was suggested to function as one of the protection strategies [3]. However, such morphology-oriented mechanism is not sufficient to fully protect the cells from the deleterious effects of UV light [3], and other mechanisms of UV protection have to be explored.

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In outdoor conditions, *A. platensis* cells are susceptible to photoinhibition [3,20,21]. Their photosynthetic activity declines when the intensity of sunlight increases and recovers under decreased sunlight intensity [26]. However, the mechanism of PSII repair has not been studied in *A. platensis*, and the role of D1 protein turnover in the repair process has not been documented. PSII repair in general is an important part of survival strategy, which involves proteolytic removal of photodamaged D1 protein via the FtsH protease both in visible [27] and UV-B light [13] followed by the coordinated insertion of newly synthesized D1 into the thylakoid membrane [28]. If photoinactivation outruns the rate of repair, the PSII pool suffers net photoinhibition leading to a decrease in photosynthetic capacity and potentially to a cell death [29–32].

In the present study we used two strains of *A. platensis* to investigate the UV-B induced impairment and subsequent repair of PSII activity in relation to changes in the amount of the D1 protein. Our results show high repair efficiency of PSII function by *de novo* synthesis of the D1 protein, which represents an important mechanism of in the amelioration of deleterious effects of UV-B radiation in *A. platensis*.

2. Materials and methods

2.1. Organism and culture conditions

Two strains of *A. platensis* were used in our experiments. These strains were: (i) strain 439, which was grown in the laboratory, had not been exposed to sunlight for decades, and was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Sciences, and (ii) strain D-0083, which has been grown under sunlight for decades in the commercial production base and was obtained from Hainan DIC Microalgae Co. Ltd., Hainan, China. Cells were grown in Zarrouk's medium in a rotary shaker at 30 °C under a 5% CO_2-enriched atmosphere and 40 μE m^{-2} s^{-1} light intensity.

2.2. UV-B treatment

Cells at mid-exponential growth phase were harvested, resuspended in fresh medium, and then dispended in open, square glasses in which 100 mL cell culture of 5 μg Chla mL^{-1} formed 1 cm layer height, maintained in suspension by magnetic agitation. UV-B radiation was provided by a Vilbert–Lourmat lamp, with maximum emission at 312 nm, in combination with 0.1 mm cellulose acetate filter (Clarfoil, Courtalouds Chemicals, UK) yielding an intensity of 4.5 W m^{-2} at sample surface. When required, lincomycin, a protein synthesis inhibitor, at a concentration of 300 μg mL^{-1} was added to the cell culture. For the recovery experiments, visible light was produced by an array of halogen spot lamps at the 40 μE m^{-2} s^{-1}. During the irradiation treatment, temperature of the culture was controlled at 30 °C by a circulator bath.

2.3. Oxygen evolution measurements

Steady-state rates of oxygen evolution were measured at 30 °C using a Hansatech DW2 oxygen electrode at a light intensity of 1000 μE m^{-2} s^{-1} in the presence of 0.5 mM 2,5-dimethy-p-benzoquinone as electron acceptor. Usually, 1 mL of cells at 5 μg Chl mL^{-1} was used in each measurement and the temperature of the culture was kept at 30 °C by a circulator bath.

2.4. Fluorescence measurements

Flash-induced increase and subsequent decay of chlorophyll fluorescence yield was measured by a double-modulation fluorometer (P.S.I. Instruments, Brno, Czech Republic) [33]. The instrument contained red LEDs for both actinic (20 μs) and measuring (2.5 μs) flashes, and was used in the time range of 100 μs–100 s. With this type of measurement, it is important to avoid distortion of the relaxation kinetics due to the actinic effect of measuring flashes. This was carefully checked, and the intensity of the measuring flashes was set to a value that was low enough to avoid reduction of Q_A in the presence of DCMU. Prior to measurements, the samples were dark adapted for 3 min.

2.5. Preparation of thylakoid membrane

A. platensis cells (25 μg) were collected by vacuum filtration of the cell suspension on a filter paper, and washed in buffer A [50 mM MES/NaOH, pH 6.5, containing 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM aminocaproic acid, and 1 mM benzamidine], and resuspended in 0.5 mL of buffer A. The same volume of glass beads (150–200 μm in diameter) was added and the cells were broken in an Eppendorf tube by vortexing three times for 60 s with 1 min interruption for cooling on ice. The unbroken cells, cell debris, and thylakoid membranes were decanted off by repeated washing of the glass beads with buffer A. The decanted material was then centrifuged at 6500 rpm for 2 min at 4 °C to remove unbroken cells and debris. The supernatant was centrifuged at 14,000 rpm for 20 min at 4 °C to obtain a pellet of thylakoids, and then the pellet was resuspended in 50 mM Tris/

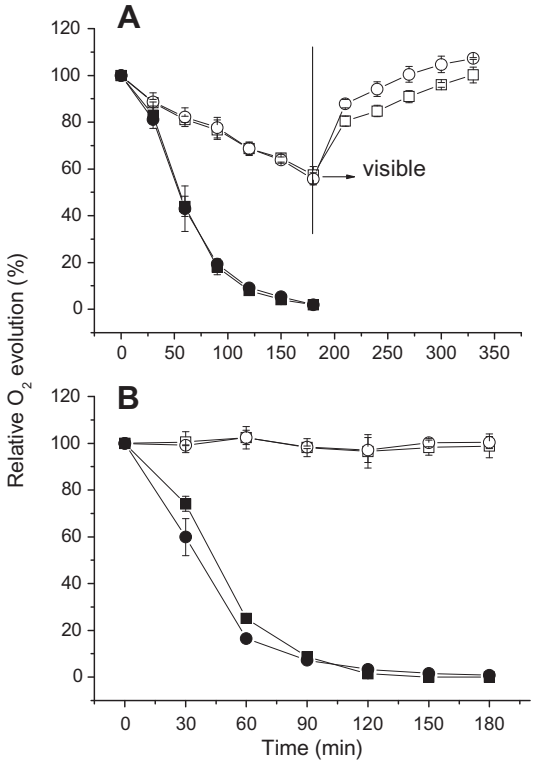

Fig. 1. Effect of UV-B light treatment on the oxygen-evolving activity of *Arthrospira platensis* cells. The experiments were performed with strain 439 (squares) and strain D-0083 (circles) either in the presence (full symbols) or absence (empty symbols) of protein synthesis inhibitor (lincomycin). (A) Cells were exposed to 4.5 W m^{-2} UV-B alone followed by a recovery period under visible light of 40 μE m^{-2} s^{-1}. (B) Cells were exposed to 4.5 W m^{-2} UV-B supplemented with 40 μE m^{-2} s^{-1} visible light. The data represents the average of three independent experiment, and shown after normalization to the oxygen evolution rates measured in the non-irradiated control cells for strain 439 (657 ± 22 μmol O_2 mg^{-1} Chla h^{-1}) and strain D-0083 (1074 ± 86 μmol $O_2$$mg^{-1}$ Chla h^{-1}).

HCl pH 7.5 and 1 M sucrose. The isolation procedure was carried out on ice in a cold room.

2.6. SDS–PAGE analysis and Western blot

Thylakoid membrane proteins were resolved by SDS polyacrylamide gel electrophoresis through 12–17% linear gradient polyacrylamide gels containing 6 M urea. 0.2–0.25 μg of Chla was loaded per lane, and the gel was run overnight at 18 °C. Proteins were transferred onto a nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Germany) by wet blotting. The D1 protein was probed with rabbit polyclonal antibody raised against the spinach D1 protein (kindly provided by Dr. R. Barbato). The primary antibody was detected by using goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Sigma). The antigen–antibody complexes were visualized by colorimetric reaction using the 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue terazolium (BCIP/NBT) system (Sigma). The bands from the scanned blots were quantified using a NIH program, Image J.

2.7. Statistical analysis

One-way analysis of variance and *t*-test were used to establish difference between two strains. A confidence level of 95% was used in all analysis.

3. Results

UV-B radiation when applied in the absence of visible light induced a gradual inhibition of the oxygen-evolving activity in *A. platensis* cells (Fig. 1A). In the absence of lincomycin, an inhibitor of protein synthesis, both strains lost about 44% of their initial oxygen-evolving activity after 180 min UV-B irradiation. In contrast, in the presence of lincomycin, both strains showed an accelerated loss of oxygen evolution under UV-B exposure. The oxygen-evolving activity was decreased to about 18% of the initial value after 90 min, and was almost completely lost in 180 min (Fig. 1A). When the UV-B-irradiated cells were transferred to visible light, both strains showed a rapid recovery of PSII activity. The rate of oxygen evolution was restored to ~90% of the original value in strain D-0083, and to ~80% in strain 439 in the first 30 min ($p < 0.05$), respectively. The oxygen-evolving activity was fully regained in the strain D-0083 after subsequent incubation for 90 min in visible light, while additional 60 min was required in the strain 439.

When *A. platensis* cells were exposed to UV-B irradiance supplemented with 40 μE m^{-2} s^{-1} of visible light, the oxygen-evolving activity was not affected (Fig. 1B). However, in the presence of lincomycin, the oxygen-evolving ability was inhibited to a greater extent than observed in the cells exposed to UV-B alone with the rate of oxygen evolution decreasing to about 8% of the initial value in 90 min, and almost completely lost in 120 min (Fig. 1A and B). The decline rate of oxygen-evolving activity was somewhat faster in the D-0083 strain as compared to the 439 strain during the first 60 min ($p < 0.005$), but was practically identical after longer illumination.

In order to further demonstrate the effects of UV-B radiation on the function of PSII, we measured the kinetics of flash-induced chlorophyll fluorescence relaxation, which reflects the reoxidation of Q_A via forward electron transport to Q_B (and Q_B^-) and back reaction with donor-side components [34,35]. In the presence of DCMU, which occupies the Q_B-binding site and inhibits the electron transfer from Q_A to Q_B, the fluorescence relaxation kinetics arises from the reoxidation of Q_A^- with donor-side components of PSII. UV-B radiation resulted in a decrease of the initial amplitude and accelerated the decay kinetics. In non-irradiated cells the relaxation follows hyperbolic kinetics with about 1s time constant, which arises from the recombination of Q_A^- with the S_2 state of the

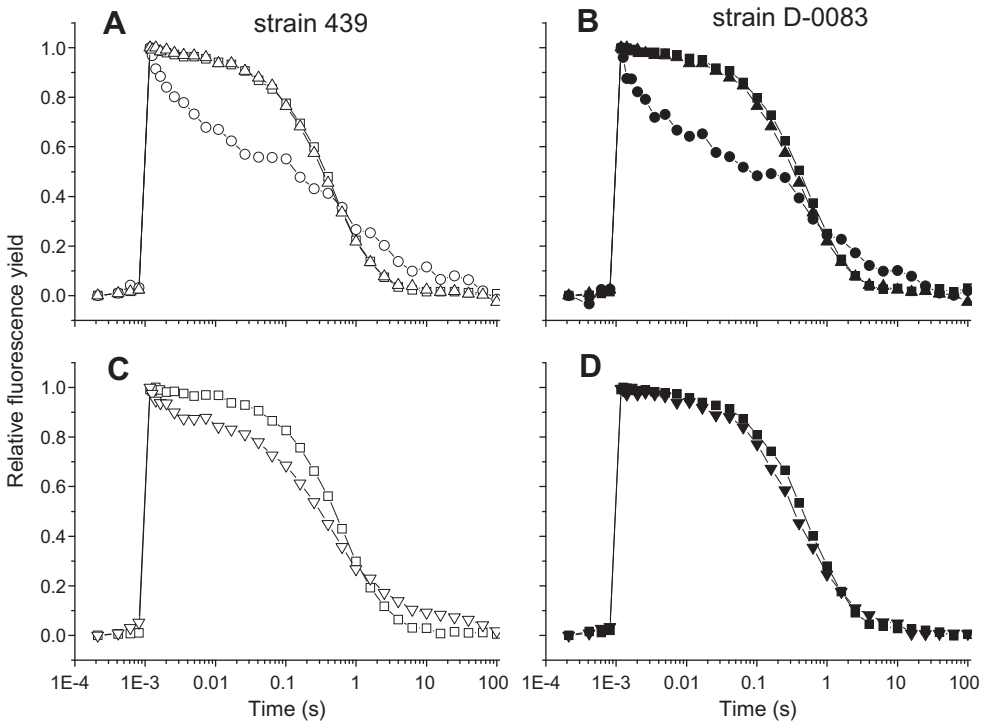

Fig. 2. Effect of UV-B light treatment on the decay of Chla fluorescence yield in *Arthrospira platensis* cells. Fluorescence decays induced by single-turnover flash were measured in the presence of DCMU. The kinetics of fluorescence relaxation are shown for cells (A and B) before (squares) and after 180 min UV-B treatment (circles) followed by 120 min recovery under visible light (up triangles), as well as (C and D) before (squares) and after 180 min combined treatment of UV-B and visible illumination (down triangles) in strain 439 (empty symbols, A and C) and strain D-0083 (full symbols, B and D). The curves are shown normalized to the same initial amplitude.

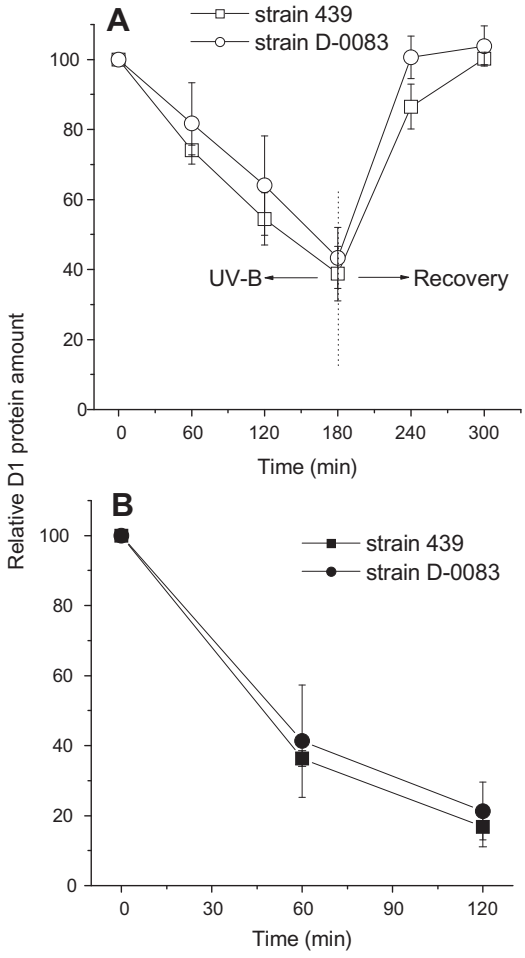

Fig. 3. Effect of UV-B light treatment on the D1 protein content of *Arthrospira platensis* cells. Cells of strain 439 (squares) and strain D-0083 (circles) were exposed to 4.5 W m^{-2} UV-B alone followed by a recovery period under visible light of 40 μE m^{-2} s^{-1} in the absence (A) or presence (B) of 300 μg mL^{-1} lincomycin. Changes in the D1 protein amount obtained from densitograms of blots of *A. platensis* thylakoid. The data are shown after normalization to the value at the 0 time point.

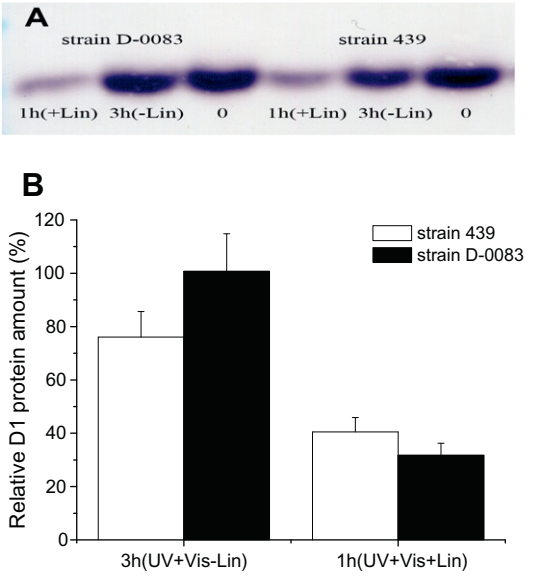

Fig. 4. Effect of UV-B plus visible light treatment on the D1 protein content of *Arthrospira platensis* cells. D1 protein content in *Arthrospira platensis* cells exposed to 4.5 W m^{-2} UV-B supplemented with 40 μE m^{-2} s^{-1} visible light. (A) Immunoblots of D1 obtained in cells of strain 439 and strain D-0083 after 0 and 3 h, exposure to UV-B plus visible light, as well as after 1 h in the presence of 300 μg mL^{-1} lincomycin, as indicated by 0, 3 h(-Lin), and 1 h(+Lyn), respectively. (B) Changes in the D1 protein amount obtained from densitograms of blots of *A. platensis* thylakoid. The data are shown after normalization to the value at the 0 time point.

water-oxidizing complex (Fig. 2A and B). After exposure to UV-B irradiation, a faster component with few ms time constant appears (Fig. 2A and B), reflecting the recombination of Q_A^- with Tyr-Z^{ox} in PSII centers in which the electron transport between the Mn cluster and Tyr-Z has been inactivated [7]. When the cells were transferred back to visible light the fast decaying phase disappeared and the decay kinetics observed in the untreated cells were restored (Fig. 2A and B), demonstrating the restoration of normal electron transfer in the PSII complex. When the cells were exposed to UV-B radiation in the presence of visible light the fast phase in the fluorescence relaxation was very small in strain 439, and was completely missing in the D-0083 strain (Fig. 2C and D).

In order to elucidate the effects of UV-B radiation on the D1 protein in *A. platensis* we followed the time course of D1 protein levels during the UV-B radiation treatment and subsequent recovery in visible light (Fig. 3A). The amount of D1 protein, in both strains, decreased during the UV-B exposure to about 40% of the initial value in 180 min. The rate of recovery was also similar in the two strains when cells were exposed to visible light although the amount of D1 appeared to be restored somewhat faster in strain D-0083 (≈100% restoration in 60 min) than in strain 439 (≈87% in 60 min) , which required another 60 min for the full recovery. In the presence of protein synthesis inhibitor, the loss of D1 protein induced by UV-B radiation was accelerated (Fig. 3B). The amount of D1 protein decreased to ≈17% of its initial level in 120 min. In contrast, the amount of D1 protein in the cells exposed to UV-B irradiation plus visible light was affected less than in those exposed to UV-B alone after 180 min exposure (Fig. 4). Strain D-0083 did not show significant changes in the amount of D1 protein, while in strain 439 it decreased to about 76% of the initial amount ($p < 0.05$). However, in the presence of protein synthesis inhibitor, the D1 protein amount lost to 40% and 31% of the initial in strain 439 and strain D-0083 respectively in 1 h exposure, but the difference between these two values was not significant ($p > 0.05$).

4. Discussion

The extent of photoinhibition depends on the balance of damage and repair reactions, which are regulated differentially by environmental conditions [36]. Self-shading produced by compressed spirals in *A. platensis* is known to play an effective role in protecting the cells from harmful UV radiation [3] by decreasing the efficiency of UV induced damage. Similar effect is exerted by the increased ratio of carotenoids relative to Chl a in the presence of UV-B due to quenching of the highly reactive oxidants produced by UV radiation [20,21]. However, the present study shows that PSII repair, which occurs via *de novo* synthesis of the D1 protein also represents an efficient defense mechanism against UV-B induced activity loss of PSII in *A. platensis*. The importance of repairing PSII activity via re-synthesis of the D1 protein during and following the UV-B exposure is demonstrated by the large acceleration of the loss of both PSII activity and D1 protein content in the presence of the protein inhibitor lincomycin (Figs. 1A, and 2, respectively). These data show that the D1 protein-related defense mechanism against UV-B at the PSII level, which has been observed in unicellular cyanobacteria such as *Synechocystis* 6803 [12], as well as in natural phytoplankton communities [37] plays also an important role in the multicellular *A. platensis*. The D1 protein is encoded

by the *psbA* gene, which often forms a small multigene family in unicellular cyanobacteria. The different copies of *psbA* genes respond differentially to UV-B exposure as was demonstrated for *Synechocystis* 6803 [12,14], *Anabeana* [14] and *Gloeobacter violeceous* [38]. This represents an important regulation of the repair process either by increasing *psbA* mRNA abundance, or by producing a more phototolerant D1 protein form. Unfortunately no specific sequence information is available so far about the *psbA* genes in *A. platensis*. Therefore, the possible role of different *psbA* genes or D1 protein forms in this multicellular cyanobacterium could not be clarified in the present investigation.

A. platensis usually grows in shallow waters either in nature or commercially operated ponds, therefore, the cells are exposed to natural sunlight in which both the UV-B and visible spectral ranges are present. The strain D-0083 had been grown under sunlight before it was collected in 2003, and cultured under laboratory conditions since then. In contrast, the strain 439 had been grown under UV-free indoor conditions for decades. Therefore, the defense mechanisms against UV radiation can differ between the two strains. This is indeed shown by the differences observed in the rate of inactivation of oxygen-evolving activity in the presence of lincomycin under combined UV-B plus visible light (Fig. 1B), in the kinetics of fluorescence relaxation under UV-B plus visible light (Fig. 2C), and also in the rate and extent of recovery of oxygen-evolving activity in visible light following UV-B light exposure (Fig. 1A). In addition, after 180 min UV-B exposure, the D-0083 strain showed higher flash-induced chlorophyll fluorescence amplitude than strain 439 (data not shown), reflecting the higher amount of functional PSII center. These results all show that the indoor 439 strain is damaged to a larger extent and repaired to smaller extent than the outdoor D-0083 strain. This conclusion is also in a qualitative agreement with the results concerning the changes of D1 protein amount (Figs. 3A, and 4) and shows that during the long term culturing under laboratory conditions part of the UV protection capacity was lost from the *A. platensis* cells. This effect seems to arise mainly from the decreased efficiency of PSII repair. However, clarification of the affected sites in the PSII repair cycle will require further investigations.

UV-B light damages primarily the Mn cluster of water oxidation in the PSII complex with additional effects on tyrosine donors and quinone acceptors [39–41]. In the presence of both visible and UV-B light, the primary inactivation of the Mn cluster occurs by UV photons, which can be followed by additional damage induced by visible light in the PSII structure [42–44]. Previous studies with intact *Synechocystis* 6803 cells showed that UV-B and visible light damage the oxygen-evolving activity of PSII independent of each other, but the two spectral ranges interact in the process of PSII repair, which results in a significant enhancement of repair capacity [42]. This effect was also seen in the present study by the prevention of UV-B induced loss of PSII activity, as well as of D1 protein amount when the UV-B radiation was supplemented by weak visible light (Figs. 1B and 4). These results demonstrate that visible light driven D1 protein repair is an important factor for protecting cyanobacteria against UV-B damage.

In conclusion, our results show that the *de novo* synthesis of D1 protein in *A. platensis* is an important defense mechanism that occurs at PSII level to counteract the UV-B damage. Visible light is not only the main source of light energy used for the photosynthesis, but also plays an important role in helping cells to restore the function of PSII that damaged by UV-B.

5. Abbreviations

D1	D1 reaction center protein of PSII
PSII	Photosystem II
UV-B	Ultraviolet-B radiation (315–280 nm)
UV-A	Ultraviolet-A radiation (400–315 nm)
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
Q_A	Primary quinone electron acceptor of PSII
Q_B	Secondary quinone electron acceptor of PSII
Tyr-Z	Tyrosine-161 or D1 protein that functions as the immediate electron donor to P_{680}^{+}

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