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Inactivation of the extrinsic subunit of photosystem II, PsbU, in *Synechococcus* PCC 7942 results in elevated resistance to oxidative stress

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Abstract PsbU is a subunit of the extrinsic complex attached to the core of photosystem II. A PsbU-mutant of *Synechococcus* PCC 7942 was isolated based on its elevated resistance to externally applied oxidative stress. PsbU-mutant exhibits fast rates of degradation of the photosystem II core protein, D1, under subsaturating as well as high-light conditions. While forward electron transfer is not affected, back electron flow is severely impaired in the mutant. We suggest that impairment of *psbU* results in production of reactive-oxygen-species, which trigger antioxidative mechanisms even under standard growth conditions. Accordingly, when challenged with external oxidative stress, these cells are more resistant than wild type cells.

Keywords: Cyanobacteria; PsbU; Oxidative stress; D1 degradation

1. Introduction

Cyanobacteria are photosynthetic prokaryotes which, similarly to green algae and higher plants, perform oxygenic photosynthesis; namely, H_2O serves as an electron donor and water breakdown yields protons and molecular oxygen. Water oxidation is a unique feature of the multisubunit protein–pigment complex, photosystem II (PSII) [1–3]. This process necessitates the production of strong oxidants within PSII and these active species may directly damage the photosynthetic reaction center [4] or lead to the production of reactive oxygen species (ROS) [5,6]. Therefore, the process of oxygenic photosynthesis dictated the development of various mechanisms allowing cells to minimize the production of ROS [7] and cope with oxidative stress, once it has occurred [8,9].

Previous studies have contributed to our understanding of the mechanisms allowing photosynthetic organisms to cope with oxidative stress [10–14]. To gain further comprehension of the cellular processes involved in ROS production and their detoxification, we selected mutants of *Synechococcus* PCC 7942 (also termed *Synechococcus elongatus*, hereafter *Synechococcus*), which exhibited higher resistance to oxidative stress compared with the wild type strain. One of these mutants was shown to possess inactivated *psbU*. This gene encodes a subunit of the lumenal complex, which contributes to the stabilization of the oxygen evolving center, a cluster of Mn, Ca and Cl ions within PSII, which is involved directly in water oxidation [15].

The subunit composition of the lumenal extrinsic complex of PSII is quite diverged between various photosynthetic organisms. PsbO, the larger subunit, is common to all organisms performing oxygenic photosynthesis. In addition to PsbO, plant complexes for example, include PsbP and PsbQ; cyanobacteria possess PsbU,V,P and Q subunits and red algal complexes possess a unique PsbQ-like subunit (PsbQ′) [15–17]. Studies of deletion mutants as well as reconstitution experiments of PSII complexes demonstrated the significance of these proteins in stabilizing the Mn cluster [18–21]. The interactions between individual subunits and their contribution to the function of the 'donor side' were studied extensively in recent years [16,22–30]. While great progress has been made in the understanding of the function of the water splitting apparatus, the role of the smaller subunits is still not well defined. This study reveals novel features of cells impaired in *psbU* and suggests a crucial role for this subunit in prevention of photodamage to PSII.

2. Materials and methods

2.1. Strains, culture conditions and isolation of mutants resistant to oxidative stress

Synechococcus sp. PCC 7942 and all strains resulting from molecular manipulations of this wild type were cultured as previously described [31]. Incandescent light (20 μmol photons m^{-2} s^{-1}) is referred to as the standard light intensity.

Synechococcus cells were mutagenized by transformation with a transposon-based inactivation library. The library was obtained by in vitro transposition of a genomic library using EZ::TN™ KAN-2 insertion kit (Epicentre, Madison, Wisconsin). Following growth of transformants in liquid growth medium in the presence of 25 μg/mL kanamycin, the cultures were plated on solid growth medium to yield a cell lawn. Drops of 10 μL of either H_2O_2 (10–50 mM) or methyl viologen (MV, superoxide producing agent, 5–50 μM) were spotted onto the mutants' cell lawn. Application of these oxidative stress-inducing agents resulted in cell-bleaching and a clear area was formed on the lawn. Such high concentrations of H_2O_2 and MV were required as *Synechococcus* cells exhibits high resistance to oxidative stress when plated at high cell density. Examination of the cleared areas under binoculars revealed the presence of single colonies. One of the mutants, which showed elevated resistance to oxidative stress, mutant 60, was chosen

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Abbreviations: chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MV, methyl viologen; PSII, photosystem II; Q_A and Q_B, primary and secondary (respectively) electron acceptor quinones of PSII; Rubisco, ribulose bisphosphate carboxylase/oxygenase; ROS, reactive oxygen species; TL, thermoluminescence

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for further analysis. Additional analysis of the oxidative stress-resistant phenotype of this mutant was done by assessing viability following application of H_2O_2 or MV in liquid culture as described previously [14]. The *katG*-mutant and the double mutant impaired in *psbU* and *katG* were produced using the construct described earlier [14].

2.2. Cloning of the genomic fragment bearing the transposon

Identification of the transposon insertion site in mutant 60 was done essentially as described earlier [32]. Briefly, genomic DNA was digested with *Pst*I and ligated to Bluescript KS. Transformants of *Escherichia coli* (DH5α) were selected in the presence of kanamycin; only the desired clones were resistant to the antibiotic due to *npt*II included in the transposon. The sequence of the genomic regions neighboring the transposon was determined using the transposon-specific primers provided with the EZ::TN™ insertion kit. The transposon was found to be inserted into the 5th codon of *psbU*.

2.3. Insertional inactivation of the gene impaired in mutant 60, psbU

A DNA fragment containing *psbU* was obtained by PCR on genomic DNA of *Synechococcus* using the primers 5′ AGGCAGAGACCGGTGTAGAGGC 3′ and 5′ ATTCAAGACGCGCTGATCGAAGGG 3′. This fragment was cloned into pGEM® T Easy vector (Promega) and a spectinomycin-resistance cassette was inserted immediately after the 35th codon using the *Nhe*I site. The resulting construct was transformed into *Synechococcus*, and clones resistant to spectinomycin were selected. PCR on genomic DNA of several transformants confirmed double homologous recombination and complete chromosome segregation (not shown). One of these clones, PsbUΩ, was selected for further analysis.

2.4. Western analysis and measurements of PSII function

Cells were broken by agitation in a bead-beater (Glen Mills, New Jersey, USA), in 10 mM Tris buffer (pH 8.0) containing protease inhibitor cocktail (Sigma–Aldrich) and fractionated by centrifugation (18 000 × *g*, 20 min). The pellet was resuspended in 0.1 M dithiothreitol, 0.1 M Na_2CO_3. Samples were separated by SDS–PAGE on a 10% gel (see legend to Fig. 4 for details). Proteins were transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad laboratories, California, USA), and probed with antibodies raised against either D1 or RbcL (AgriSera, Vännäs, Sweden). The anti-D1 antibodies detect both form 1 and 2 of the protein [33]. Horse radish peroxidase conjugated rabbit anti-chicken IgY or goat anti-rabbit IgG (Sigma–Aldrich) were used as secondary antibodies for D1, and RbcL, respectively. Super-Signal West Pico Chemiluminescent Substrate (Pierce) was used for detection of the secondary antibodies.

Oxygen evolution in vivo was measured using a Clark type electrode with 40 μM 2,6-dimethoxy-1,4-benzoquinone (DMBQ) and 250 μM potassium ferricyanide as electron acceptors. Fluorescence was measured by an FL200 fluorometer (PSI, Brno, Czech Republic) [34]. Q_A^- decay measurement data was fitted to a third order exponential decay. Thermoluminescence (TL) measurements were performed on cells prepared as described in Carpenter et al. [35], using a home made apparatus [36]. Samples containing 20 μgr chl were dark adapted for 3 min at room temperature. Following dark adaptation, the temperature was dropped to −80 °C and 10 saturating flashes were applied. The TL signal was measured during constant heating from −80 to 60 °C.

Photoinhibition was performed on 40 mL cultures (containing 5 μg chl mL^{-1}) at a constant temperature of 30 °C. Illumination at 500 μmol photons m^{-2} s^{-1} was provided using a heat- and UV-filter. At the indicated times, samples were removed for O_2 evolution, and F_v/F_m measurements as well as D1 analysis. F_v/F_m measurements were performed using apparatus PAM101/102/103 (Walz, Effeltrich, Germany).

Data shown in this study represent one of at least three independent experiments except for data presented in Figs. 3A and 5A, which show averages of measurements on three independent cultures.

3. Results

3.1. Inactivation of psbU results in elevated resistance to exogenously applied oxidative stress

Synechococcus mutants raised by random transposon insertion were selected for their ability to survive H_2O_2 concentrations deleterious to the wild type strain (see Section 2 for details). Molecular analysis of one of these mutants revealed that the transposon was inserted into the coding region of *psbU*, encoding a subunit of the lumenal complex of PSII. Directed inactivation of *psbU* resulted in a strain designated PsbUΩ, which also exhibited resistance to oxidative stress. Similarly to *psbU* mutants of various organisms [22,24], *Synechococcus* PsbUΩ also exhibited slower growth rate when limited for Ca^{2+} or Cl^- (not shown). Fig. 1 shows assessment of viability of the wild type strain and PsbUΩ following treatment with oxidative stress-inducing agents. The mutant exhibits increased resistance to H_2O_2 (Fig. 1A) as well as to the superoxide producing agent, MV (Fig. 1B), compared to the wild type strain. It is noteworthy that cell density affected the H_2O_2 and MV concentrations the strains could tolerate; at higher densities cells survived higher concentrations of the toxic compounds. Nevertheless, the relative differences between the wild type and PsbUΩ were observed at all cell densities.

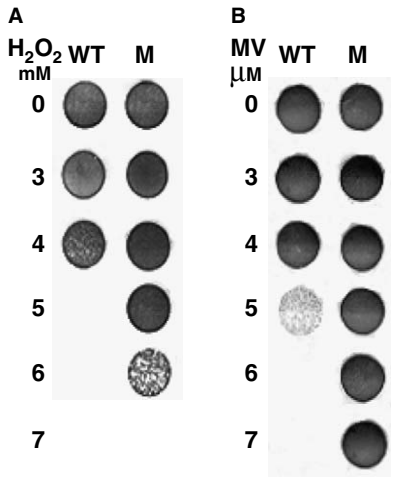

Fig. 1. Viability of *Synechococcus* (WT) and its PsbU-mutant (M) following treatment with H_2O_2 (A) or MV (B). Liquid cultures were treated with the indicated concentrations of the oxidative-stress inducing agents. Aliquots of 5 μL were spotted on solid growth medium.

It has been previously shown that the ability of cyanobacterial cells to survive high concentrations of externally applied H_2O_2 relies on the activity of KatG [14,37], an enzyme belonging to the group of prokaryotic catalase-peroxidases. To test if the resistance to oxidative stress exhibited by PsbUΩ stems from elevated KatG activity, we compared the ability of the mutant and the wild type strains to detoxify added H_2O_2. PsbUΩ hydrolyzed 6 mM (Fig. 2A) and 10 mM (not shown) H_2O_2 faster than the wild type strain.

katG-mutants of *Synechococcus* are devoid of catalase activity [14]; presumably, this is the only catalase expressed under the growth conditions used in our studies. As KatG provides the dominant activity for detoxification of externally added H_2O_2, *katG*-inactivated strains allow assessment of H_2O_2 detoxification by cellular peroxidases. When a *katG*-mutant and the double mutant impaired in *psbU* and *katG* were exposed to 30 μM H_2O_2, a faster rate of detoxification was observed in the case of the double mutant (Fig. 2B). Taken together, the detoxification analyses of the various strains imply that inactivation of *psbU* resulted in elevated activity of KatG as well as higher activity of cellular peroxidase(s).

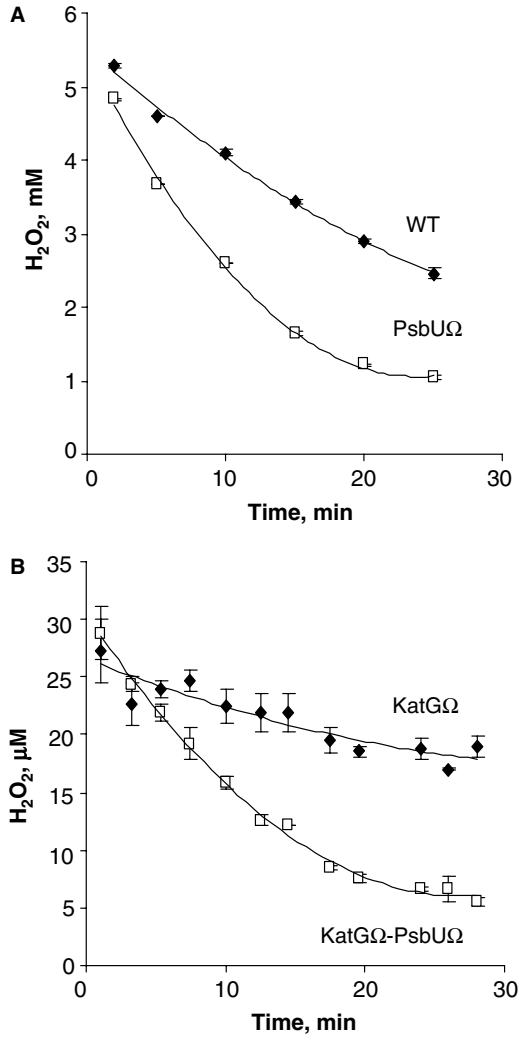

Fig. 2. Detoxification of added H_2O_2 by wild type (WT) and PsbUΩ (A) and their cognate catalase mutants, KatGΩ and KatGΩ-PsbUΩ (B). 6 mM and 30 µM H_2O_2 were added at time zero in (A) and (B), respectively.

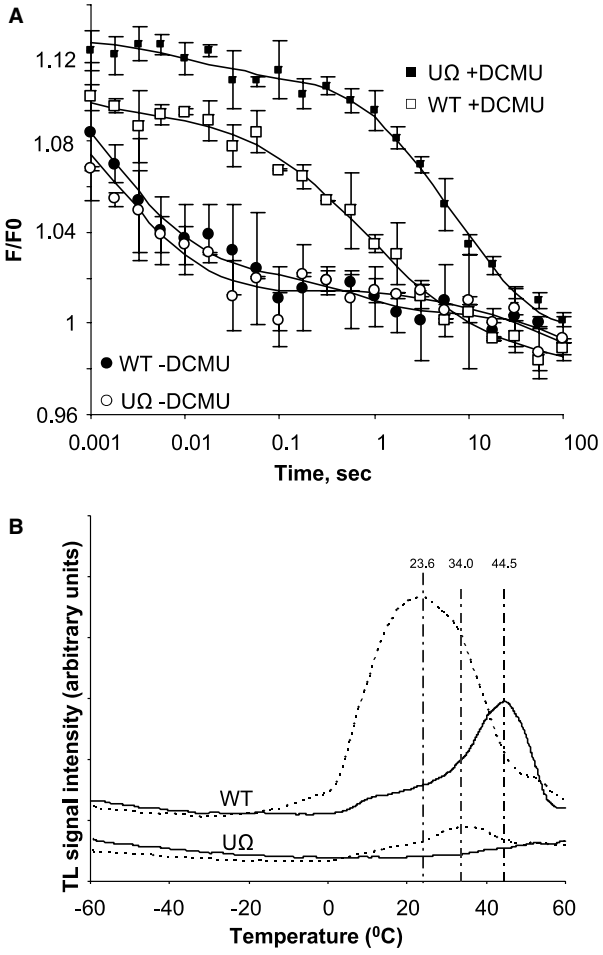

Fig. 3. $Q_A^{\bullet-}$ relaxation fluorescence kinetics (A) and TL (B) of wild type (WT) cultures and PsbUΩ (UΩ). 5 µM DCMU was added where indicated in (A); dashed lines in (B) indicate the presence of DCMU.

3.2. Effect of inactivation of psbU on PSII function

The *Synechococcus* PsbUΩ mutant exhibits maximal PSII oxygen evolution rates that are practically identical to those of wild type (330 ± 37 and 334 ± 42 µmol O_2 mg chl^{-1} h^{-1}, respectively, $n = 3$). The PSII yield parameter, F_v/F_m [38], is 0.68 ± 0.02 and 0.60 ± 0.01 for wild type and mutant cultures, respectively ($n = 3$). Both parameters indicate normal forward electron transfer capacity of PSII under standard growth conditions.

While forward electron transfer in PSII was virtually similar between the wild type and the PsbUΩ strains, a marked difference in the rate of back electron transfer was observed in the mutant. The fluorescence kinetics data presented in Fig. 3A track the reduction state of Q_A following a saturating light pulse given at time zero. Subsequent weak measuring pulses were used to probe the reduction state of Q_A in the PSII population in the sample as affected by electron transfer. In the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) electrons can flow forward to the Q_B site, or through back electron transfer to Mn clusters in the $S_{2,3}$ states. In the presence of DCMU, only back electron transfer reactions occur. The rates of $Q_A^{\bullet-}$ re-oxidation in the absence of DCMU are similar in wild type and PsbUΩ cultures. In the presence of DCMU, however, the rate of $Q_A^{\bullet-}$ re-oxidation is slowed down significantly in mutant cultures. The time constant of the exponential decay fit is 1.2 ± 0.02 s for wild type cultures and 6.1 ± 0.84 for PsbUΩ cultures ($n = 3$).

In order to look into the energetics of back electron transfer reactions in further detail, TL analysis was performed (Fig. 3B). To ensure maximal trapping efficiency of charge separated donor/acceptor pairs, excitation was provided at −80 °C. The typical $Q_B^{\bullet-}/S_{2,3}$ peak at 44.5 °C (in the absence of DCMU) and $Q_A^{\bullet-}/S_2$ peak at 23.6 °C (in the presence of DCMU) [39], were observed in wild type cultures. In PsbUΩ cultures, TL signal was undetectable in the absence of DCMU. In the presence of DCMU, the signal intensity was only 12% of the wild type signal. In addition, the peak position was shifted by +10.4 °C, indicating higher activation energy for the back electron transfer reactions giving rise to the TL glow curves [40].

3.3. Inactivation of psbU results in rapid degradation of the central PSII protein, D1

It is well established that the PSII reaction center protein, D1, is constantly damaged, degraded and replaced with a

newly synthesized protein [4]. Therefore, addition of chloramphenicol, a protein synthesis inhibitor, allows for the examination of the degradation rate of the D1 protein.

D1 specific antibodies were used to follow the amount of the protein in the different strains grown under standard conditions (20 μmol photons m^{-2} s^{-1}). In the presence of chloramphenicol, PsbUΩ exhibits a substantially faster decrease in the amount of D1 as compared to wild type cells (Fig. 4A and B, see figure legend for experimental details). In the absence of the inhibitor, however, PsbUΩ and the wild type strain contain comparable levels of D1, indicating that the repair process in the mutant is capable of maintaining a normal steady state level of D1. The use of antibodies against the large subunit of ribulose bisphosphate carboxylase/oxygenase (Rubisco) reveals a constant level of the protein (Fig. 4C), indicating that the observed fast degradation of D1 in PsbUΩ does not reflect a general problem of protein stability.

3.4. Inactivation of psbU renders PSII more susceptible to photodamage

The fast degradation rate of the D1 protein in PsbUΩ under relatively low light conditions, deduced from the inhibition studies using chloramphenicol, encouraged us to examine PSII function in the mutant under high light intensity. When illuminated with 500 μmol photons m^{-2} s^{-1} wild type cells exhibit a gradual decrease in F_v/F_m throughout the duration of the experiment (from 0.68 to 0.42 following 60 min of the high light treatment). PsbUΩ exposed to the same light conditions exhibits a dramatic decrease with time in F_v/F_m values (from 0.60 to 0.20, Fig. 5A).

Examination of D1 in the course of the high-light illumination indicated decrease in D1 level in the wild type as well as PsbUΩ. The mutant, however, exhibits a much faster decrease in D1 level as compared to the wild type strain; in fact, the D1 protein was barely detectable following 60 min of the high light treatment in the absence of chloramphenicol (Fig. 5B). It may therefore be suggested that in the case of the mutant, the rate of damage to PSII, and the consequent degradation of D1, exceeds the capacity of the repair machinery under these light conditions.

Oxygen evolution rates of low light grown wild type and mutant cells are comparable (see above). Following 15 min of high light treatment oxygen evolution from mutant cells could no longer be detected while wild type cells exhibited 70% of the initial rate of oxygen evolution. Thereafter, oxygen evolution from wild type cells gradually degreased to 50% and 20% of the initial rate following 30 and 60 min of the high light treatment, respectively.

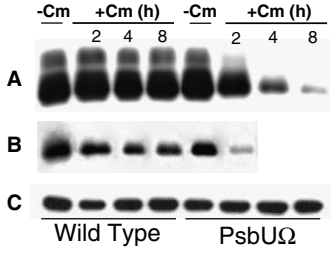

Fig. 4. Western analysis of cellular extracts of wild type and PsbUΩ using antibodies to the PSII-core protein, D1 (A and B) and to the large subunit of Rubisco (C). The anti-D1 antibodies detect both form 1 and 2 of the protein [33]. Where indicated, chloramphenicol (Cm, 250 μg/mL) was added for the indicated time (h). Panels A and B show analyses of pelletable fractions (1 and 0.2 μg chl per lane, respectively) whereas in C soluble fractions were analyzed (5 μg protein per lane).

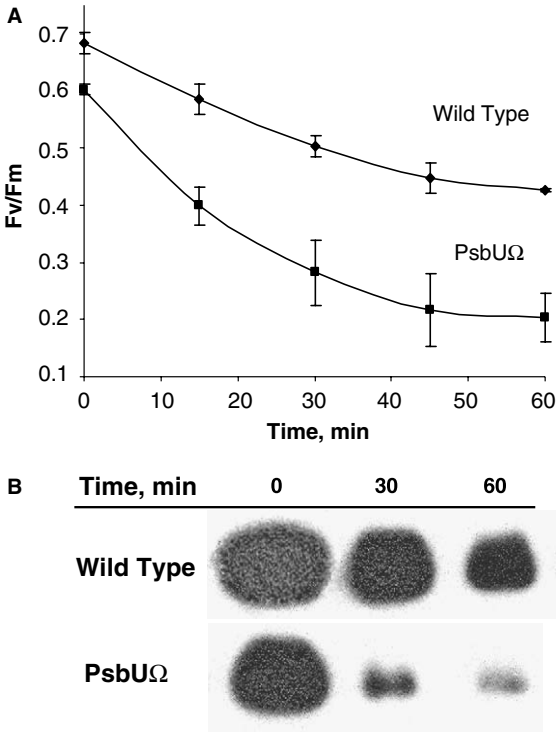

Fig. 5. F_v/F_m (A) and Western analysis using antibodies to D1 (B) in wild type and PsbUΩ cultures illuminated with 500 μmol photons m^{-2} s^{-1} for the indicated time. Data shown in (A) are averages and standard deviation of measurements on three independent cultures. Western analysis was performed on whole cell extracts (1 μg chl per lane); a single representative experiment out of three is shown.

4. Discussion

The role of PsbU, a subunit of the lumenal complex which contributes to the stabilization of the Mn, Ca and Cl cluster of ions, was studied in several organisms. It has been shown that PsbU optimizes the availability of Ca^{2+} and Cl^- cofactors required for PSII function [22,24,25]. Additionally, PsbU contributes to the thermostability of PSII [28,41].

This study reveals a novel phenotype associated with inactivation of PsbU; the *Synechococcus* PsbU-mutant originated from a screen for mutants exhibiting elevated resistance to externally applied oxidative stress (Fig. 1) and accordingly, the mutant is characterized by increased activity of catalase (Fig. 2A). Furthermore, comparison of catalase inactivated strain, KatGΩ, and the double mutant KatGΩ-PsbUΩ indicated that inactivation of PsbU resulted in elevated activity of cellular peroxidase(s) (Fig. 2B).

Evaluation of PSII forward electron transfer reaction by measuring F_v/F_m and maximal oxygen evolution rate does not indicate any major difference between wild type and mutant cultures. Analysis of *Synechocystis* PCC 6803 PsbU-mutant, however, indicated slightly lower oxygen evolution rate as well as F_v/F_m ratios, as compared to its cognate wild type [24]. Nevertheless, the overall effect of the absence of the PsbU

protein on photosynthetic activity was minor as compared to the effect of the major donor side component PsbO [23].

In contrast to PSII function associated with forward electron transfer, measurements that reflect back electron transfer ($Q_A^{\bullet-}$ re-oxidation as well as TL data) were found to be substantially different in the case of *Synechococcus* and its PsbU-mutant (Fig. 3). $Q_A^{\bullet-}$ re-oxidation rates were about 5-fold slower in the mutant (Fig. 3A). TL signal could not be detected in the mutant unless DCMU was present (Fig. 3B). This signal intensity was, however, 10-fold lower and the peak temperature was up-shifted by 10 °C, compared to wild type cells (Fig. 3B). Different results were reported for *Synechocystis* PCC 6803 in which substantial TL signal was observed in the absence of DCMU. Furthermore, signal intensity was similar to that of the wild type and the peak-temperature was shifted by only +4 °C [22]. It should be considered that insertional inactivation of *psbU* may result in further loss of subunits of the donor side of PSII. For example, isolated PSII complexes of *Synechocystis* PCC 6803 PsbU mutant contain reduced amounts of psbO, *Q*, and *V* [24]. Therefore, one should not exclude the possibility that, in this study, the observed phenotype of PsbU-mutants results from loss of multiple donor side subunits.

Importantly, our study reveals a new feature of impairment of *psbU*; the mutant is characterized by an accelerated rate of D1 degradation under high light illumination as well as during growth at sub-saturating light conditions. D1 degradation serves to eliminate damaged protein from PSII reaction center [4]. The faster D1 degradation in the mutant (Figs. 4 and 5) may therefore suggest a higher rate of damage to the reaction center core of PsbUΩ. Furthermore, PSII activity under high photon flux decreases in the mutant at a much faster rate than the wild type cells exposed to the same experimental conditions. Hence, it may be speculated that the unusual back electron transfer in the *Synechococcus* PsbU-mutant (Fig. 3) results in the increased light sensitivity. While interpreting the TL data one should bear in mind that a number of additional recombination pathways have been suggested for PSII, and that only charge recombination events occurring through the $P680^+/Pheo^-$ pathway are reflected in the TL measurement [42,43]. In the case of the mutant, in the absence of DCMU we do not measure any TL signal (Fig. 3B). Since energy trapped in the charge-separated state has to dissipate, one has to assume that the routes for energy dissipation in this case do not yield a TL signal. It may be suggested that these modes of charge recombination are associated with elevated levels of damage to the PSII reaction center (as indicated by D1 degradation under relatively low – as well as high-light intensity; Figs. 4 and 5, respectively). Though speculative, it is possible that due to the slow kinetics of charge recombination observed in PsbUΩ (Fig. 3A) long lived cation radicals such as $P680^+$ and Yz^+ may cause damage to PSII.

A noteworthy recent study has shown light sensitivity of a PsbU-mutant of *Synechocystis* PCC 6803 [24]. It will be interesting to examine D1 degradation as well as resistance to oxidative stress in this mutant.

In vitro studies have documented the production of H_2O_2 by PSII centers depleted of the extrinsic lumenal protein complex [44]. The elevated rate of D1 degradation in *Synechococcus* PsbUΩ reported here indicates a higher rate of photodamage even under moderate light intensity (20 μmol photons m^{-2} s^{-1}, Fig. 4). Possibly, the higher rate of damage to D1 in PsbUΩ stems from modifications of the protein structure around the Mn cluster of the water-oxidizing complex, which result in oxidative damage. Taken together, it may be suggested that impairment of *psbU* results in the production of ROS, which trigger antioxidative mechanisms under standard growth condition. Accordingly, when challenged with external oxidative stress, these cells are already equipped with defense mechanisms and therefore are more resistant than wild type cells (Figs. 1 and 2). Nevertheless, these elevated antioxidative activities cannot protect PSII of the mutant. By contrast, inactivation of *psbU* renders PSII more susceptible to damage compared to the wild type, as reflected by photoinhibition experiments and the higher rate of D1 degradation.

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