Characterization of a phototolerant mutant of *Synechocystis* sp. PCC 6803 created by random mutagenesis of psbAII gene

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Photosensitivity and photosynthetic characteristics have been analyzed in wild type (KC) and its psbAII mutant (16) of *Synechocystis* having three point amino acid substitutions, i.e., N322I, I326F and F328S, which are localized in the C-terminal extension of D1 protein of the photosystem II reaction center. Wild type and mutant cells show almost an identical growth pattern under normal/low light (30 μmol m−2 s−1, 30°C) liquid culture (BG-11) condition. However, upon shifting the cultures to high light (500 μmol m−2 s−1, 30°C), these two types of cells exhibit entirely different growth characteristics, i.e., the mutant cells continue to grow normally whereas, the control cells fail to adapt to the light stress and eventually resulting in complete loss of the photosynthetic pigments. On the other hand, a quick loss in the Fv/Fm value with half - decay time of about 30 min is observed in the mutant, in contrast to 120-130 min in case of control, upon shifting to high light conditions. In spite of this, mutant cells are able to adapt and grow well under prolonged high light exposure even after losing a major part of the variable yield of chlorophyll fluorescence (Fv/Fm). The high light treatment also induced decrease in the level of D1 protein in the mutant. However, half - decay time for D1 is much longer (~10 hr) than that of variable fluorescence. Thus, the mutant cells have shown an unique way for cell growth and maintenance under high light even after losing Fv/Fm and photosynthetic oxygen evolving capacity as well as D1 content to a great extent. Therefore, these results could extend an interesting insight to understand the coordination of physiological, biochemical and molecular mechanisms regulating phototolerance of the photosynthetic organisms.

The D1 protein is encoded by psbA gene which exists as a single copy gene on the plastid genome in higher plants and algae while, it belongs to a small multigene family in cyanobacteria with two to seven genes. In cyanobacterium *Synechocystis* PCC6803 psbA has three variant forms, namely, *psbA*, *psbAII* and *psbAIII* (ref.1-3). However, *psbAII* gets rarely expressed while other two forms i.e., *psbAIII* and *psbAIII* share 99% nucleotide identity and also encode an identical D1 protein product. Interestingly, *psbAII* accounts for almost 90% of the produced psbA transcript. The photosystem II reaction center consists of two structurally similar proteins D1 and D2 along with cytochrome b559 (ref.6). D1 and D2 polypeptides exist as hetero-dimers within the thylakoid membrane associated with major components that mediate primary charge separation and water oxidation. D1 protein is highly light sensitive in contrast to other photosynthetic reaction centre core proteins. It possesses a rapid turn-over potential under strong irradiance and also plays an important role in energy transformation. The inherent rapid turn-over tendency of D1 protein sustains photosynthetic efficiency under photoinhibitory irradiance. Several investigations have already been made in the last few years to reveal the molecular mechanism of photoinhibition which enables photosynthetic organisms to cope up with strong photon flux densities (PFDs) of photosynthetic oxygen evolving capacity as well as D1 content to a great extent. Therefore, these results could extend an interesting insight to understand the coordination of physiological, biochemical and molecular mechanisms regulating phototolerance of the photosynthetic organisms.

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terminal portion of the D1 polypeptide in *Synechocystis* PCC 6803, which form blue-green colonies phenotypically, if placed under low or strong PFDs. The phenotypic appearance gets changed in wild type cells under high irradiance due to photobleaching of their light-harvesting pigments, having unaltered D1 polypeptide. Consequently, it appears that mutational consequences in D1 protein have extended phototolerance to the mutant cells with the ability to retain photosynthetic pigments and unaffected spectral properties. The mutant showing phototolerance has triple amino acid substitutions viz., N322I, I326F and F328S, all localized in the E-loop of the D1 polypeptide, near C-terminal extrusion, oriented towards luminal side of the thylakoid membrane. It was therefore chosen to elucidate the integrated physiological, biochemical and molecular means regulating phototolerance under strong illumination.

Materials and Methods

**Experimental organism and culture conditions**

The cyanobacterium *Synechocystis* PCC 6803 having multiple copies of *psbA (psbAl, psbAll and psbAllI)* gene was chosen as an experimental organism. *Synechocystis* PCC 6803, Cm 4‡-1 abbreviated as KC (control strain) was kindly gifted by Dr Debus, UC, Riverside, USA and maintained in Prof. Kimi-yuki Sato’s laboratory, having *psbAl* and *psbAllI* gene forms inactivated by insertion mutagenesis using antibiotic cassettes. The various *psbAll* mutants from control KC strain were generated by random PCR. Mutant 16 was selected to find out *psbAll* mutational consequences on photosynthetic characteristics favouring phototolerance. The mutant has shown three point amino acid substitutions i.e., N322I, I326F and F328S as confirmed by *psbAll* gene sequencing through colony PCR (Table 1). The wild type (KC) and its *psbAll* mutant were grown on either BG11 solid (Bacto-agar 1.5%, Difco) or in BG11 liquid cultures, always containing antibiotics kanamycin (5 µg/ml), spectinomycin (5 µg/ml) and chloramphenicol (2.5 µg/ml). The solid BG11 medium was supplemented with 1.5 % (w/v) agar, 10 mM TES-KOH pH (8.0) and 0.3% (w/v) sodium thiosulphate. BG11 liquid culture contained 5 mM HEPES-NaOH (pH 7.5). The host strain KC and mutant 16 cells were grown in liquid BG11 at 30°C with continuous bubbling by using sterile atmospheric air containing 1% CO2 supply for efficient mixing of the growing cells.

<table>
<thead>
<tr>
<th>Cyanobacterium strain</th>
<th>Nature of substitutions</th>
<th>Amino acid</th>
<th>Nucleotides</th>
<th>Photo-sensitivity</th>
</tr>
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<tr>
<td>Wild Type (KC)</td>
<td>Unaltered D1 polypeptide</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mutant (16)</td>
<td>N322I (Asn→Ile)</td>
<td>AAC-ATC</td>
<td></td>
<td>Phototolerant</td>
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<tr>
<td></td>
<td>I326F (Ile→Phe)</td>
<td>ATC-TTC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>F328S (Phe→Ser)</td>
<td>TTC-TCT</td>
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The cell cultures were illuminated with low PFDs (white fluorescent bulb, 30 μmol photons m-2 s-1) or high light (heat filtered halogen lamp, 500 μmol photons m-2 s-1). For all studies cells were grown as a continuous culture to obtain mid-logarithmic growth phase (A750=0.8) unless mentioned otherwise.

Absorption and chlorophyll measurements

The cell turbidity was monitored spectrophotometrically (Milton-Roy, Spectronic 3000) by measuring optical density at A750 nm in a cuvette with 1 cm light path. Chlorophyll content of the cells and thylakoids was estimated as by using 80% acetone.

In vivo photoinhibitory treatment

*Synechocystis* sp. PCC 6803 cells were harvested (5000 g, 10 min at room temperature) at mid-logarithmic growth phase (A750=0.8) and resuspended in BG11 medium to a final concentration of 10 μg chl/ml. These cells were illuminated under PFDs of 500 μmol photons m-2 s-1 using halogen lamp as a light source. The temperature was controlled so as not to exceed 30°C and aliquots for the various measurements were withdrawn at specific time period. After photoinhibitory treatment both control and mutant cells were immediately re-kept under growth PFDs (30 μmol m-2 s-1 or darkness at 30°C) to allow recovery process. The photoinhibition was monitored by measuring oxygen evolving capacity and fluorescence induction kinetics (Fv/Fmax).

In vivo measurements of oxygen evolution

The oxygen evolution was measured under saturating light with an oxygen electrode (Hansatech) at 25°C using light projector (Hilux–HR 210, Japan) as a light source. All measurements were made by using 2 ml cell suspension corresponding 10 μg chl/ml
under saturating white light (3000 µmol photons m⁻²s⁻¹). The measurements were performed in fresh BG-11, either without artificial electron acceptor or in the presence of 1 mM dichloro-p-benzoquinone (DCBQ), 1 mM dimethyl-p-benzoquinone (DMBQ) with 3 mM potassium ferricyanide to keep the quinones in the oxidized form.

Preparation of cell extracts and thylakoid membranes
Cyanobacterial cells were harvested (10,000 g, 10 min at 4°C), and the pellet was resuspended in thylakoid buffer (1/100 of the original culture volume) containing 20 mM MES-NaOH (pH 6.5), 5 mM MgCl₂, 5 mM CaCl₂ and 25% glycerol (w/v). Cell suspension (~1.0 ml) was transferred in ice-cooled screw-cap tube (2 ml) containing glass beads (1.5 g). The cells were broken using Mini Bead Beater (Bio-Spec Product, USA) by six breaking cycles (20 sec shaking at 5000 rpm), each followed by 3-5 min cooling on ice. The broken cells along with debris and glass beads were transferred into a screw-cap plastic tube (50 ml) and then diluted by adding thylakoid buffer (5 ml), mixed gently and subsequently supernatant was collected in a centrifuge tube. This was repeated three times to extract thylakoids. After, centrifugation (2000 g, 10 min at 4°C) to remove unbroken cells and cell debris, the suspension was further centrifuged (15,000 g, 30 min at 4°C) to get purified thylakoid pellet. The pellet was suspended in minimum thylakoid buffer containing CaCl₂ (20 mM), and used for Western blot analysis.

SDS-PAGE and Immunodetection of D1 protein
Thylakoid membranes were isolated as described earlier. The polypeptides of the thylakoid membranes equivalent to 2 µg of chl were fractionated on SDS-polyacrylamide gel (16%), containing urea. The fractionated polypeptides were transferred onto 0.2 µm nitrocellulose membrane (Schleicher and Schuell) using semi-dry blotting apparatus (Atto, Japan) for 90 min at 200 mA constant current. The blots were probed with antibody specific for the primary D1 protein (kindly gifted by Prof. Ikeuchi, Tokyo University, Japan) followed by secondary anti-rabbit antibody (Amersham), subsequently treated by chemiluminescence kit (ECL, Amersham) to detect signals on X-ray film (Fujifilm, Japan). The D1 protein was quantified by scanning the X-ray immunoblots with laser densitometer (Molecular Dynamics, PD110).

Chlorophyll fluorescence measurements
The measurements of Fv/Fmax from Synechocystis cells were made with PEA (Plant Efficiency Analyzer, Hansatech, UK). For recovery experiment cells were photo-inhibited to about 50% of the Fv/Fmax (variable fluorescence over maximum fluorescence) value at the beginning of the experiment. This was accomplished in ~30 and ~120 min with mutant and control cells respectively, after imposing photo-inhibitory treatment (500 µmol m⁻² s⁻¹, 30°C). The 2 ml cell suspension was used in BG11 culture medium containing 10 µg chl for all measurement, after 15 min dark adaptation at 30°C, which allow relaxation of fast fluorescence-quenching components.

Results
Growth characteristics
The growth rates and phenotypic appearance of mutant (f6) having three point mutations i.e., N322I, I326F and F328S, localized in the E-Ioop of the D1 polypeptide (Table 1) and its control (KC) cells were almost identical (Fig. 1A and Table 2) under normal growth light (30 µmol photons m⁻² s⁻¹, 30°C). In spite of similar growth trends, wild type cells showed higher photosynthetic efficiency (Fv/Fmax), oxygen evolving capacity along with D1 content (Table 2). However, both cell types showed typical blue-green colour under BG 11 liquid/solid culture medium, if grown under low light. While, blue-green colour of the wild type (KC) cells get photo-bleached quickly upon exposure to high irradiance (500 µmol m⁻² s⁻¹, 30°C), mutant cells did not show any sign of photo-bleaching and grew well continuously, even if placed for a longer duration under strong light (Fig. 1B). Despite displaying a yellow-green colour, the wild type cells exhibited a slightly faster growth rate in the beginning of high light illumination. However, these cells were not able to sustain their growth rate because of severe photooxidation of the PBS and chlorophyll as well. But, these photobleached cells did not lose their viability, as confirmed by placing them back under normal growth light (data not shown). In contrast to this, mutant cells started with slow rise-up followed by continuous increase in their growth trend (Fig. 1B) with the ability to retain photosynthetic pigments. These observations are also supported by unaffected absorbance spectra of mutant cells while, dramatic decline in the absorbance at A620nm has been observed in wild type cells due to loss in the major light-harvesting pigment protein complex i.e., PBS (data not shown).
Photoinhibition of PSII in vivo

The oxygen evolving activity was monitored for the wild type and mutant cells at mid-logarithmic growth phase ($A_{730} = 0.8$). These cells (10 µg chl/ml) were subjected to photoinhibitory treatment for two hours. Subsequently, their oxygen evolution patterns were measured by using DCBQ or DMBQ as an artificial electron acceptor. Nearly, 50% higher oxygen evolution ($H_2O$-DCBQ) values were found in control cells with almost identical whole chain electron transport activity as monitored in the absence of electron acceptor if grown under normal growth irradiance. The photoinhibitory irradiance induced about 50% inhibition of PSII activity in wild type cells in 140 min whereas, it happened in four times less duration (30-33 min) in the mutant cells (Table 2). A rapid declining tendency of PSII activity has also been adapted throughout the photoinhibitory illumination by mutant cells while, wild type cells followed a gradual and slower declining pattern. Consequently, about 60% and 20% PSII activity was maintained after two hour of the photoinhibitory treatment in KC and mutant 16 cells respectively, as measured by using an artificial electron acceptor (DCBQ) to monitor the status of photoinhibition at specific time intervals (data not shown). Generally, DCBQ has been observed as a better electron acceptor (Table 3), but it also becomes limiting for stimulating electron transport with mutant cells particularly, if treated long time under strong light. This indicates towards $Q_A$ perturbation, while DMBQ reflects inhibition for

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\text{Table 2—Photosynthetic characteristics of wild type (KC) and its \textit{psbAII}/D1 mutant strain (16) of cyanobacterium \textit{Synechocystis} PCC 6803} \ [\text{Values in parenthesis are the mean values of fluorescence variable/fluorescence maximum ratio (Fv/Fmax) obtained from at least 5-7 experiments using different cultures]}.
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| Strain/ | Doubling | Fv/Fmax | $^{\text{a}}$PI =50% |
| mutant | time or | | |
| (hr) | Autotrophic | | |
| KC | DCBQ | DCBQ | DCBQ |
| Wild type | 16-18 | 0.550 (100) | 125 ± 5 | 140 ± 10 |
| Mutant (16) | 16-18 | 0.400 (73) | 30 ± 2 | 32 ± 3 |

$^{a}$Cells grown up to the mid-log phase ($A_{730} = 0.8$) under growth light (30 µmol m$^{-2}$s$^{-1}$) were shifted under photoinhibitory irradiance (500 µmol m$^{-2}$s$^{-1}$) to create ca. 50% photoinhibition. The temperature was maintained at 30°C throughout.

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\text{Table 3—Steady state rates of oxygen evolution from wild type (KC) and D1 mutant (16) cells of \textit{Synechocystis} PCC 6803 grown under normal growth conditions (30 µmol m$^{-2}$s$^{-1}$, 30°C and 1% CO$_2$ mixed with air) [The rates are shown in arbitrary units using the 100 value for the rate from wild type cells without adding acceptors (210 ± 15 µmole O$_2$ mg$^{-1}$ chl hr$^{-1}$). Values in parenthesis are the per cent values of the oxygen evolution rates of mutant cells (16) relative to that of the wild type (KC) in presence or absence of same acceptor (either DCBQ or DMBQ with FeCy). All measurements were made under saturating white light (3000 µmol m$^{-2}$s$^{-1}$) at 25°C. The data is based on three to five independent experiments using different cell cultures].}
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<table>
<thead>
<tr>
<th>Nature of O$_2$ evolution</th>
<th>Wild type (KC)</th>
<th>Mutant (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100 (100)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>1 mM DCBQ + 3 mM FeCy</td>
<td>270 (134)</td>
<td>270 (134)</td>
</tr>
<tr>
<td>1 mM DMBQ + 3 mM FeCy</td>
<td>130 (90)</td>
<td>130 (90)</td>
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Fig. 1—Growth characteristics of wild type (KC) and its \textit{psbAII}/D1 mutant strain (16) of cyanobacterium \textit{Synechocystis} PCC 6803. (A): Cells grown under low light (30 µmol m$^{-2}$s$^{-1}$). (B): Cells grown under low light up to the early log phase ($A_{730} = 0.35$) and then shifted under high light (500 µmol m$^{-2}$s$^{-1}$) The cultures were supplied sterile atmospheric air mixed with 1% CO$_2$ for continuous stirring. Temperature was maintained at 30°C throughout. Cell growth was monitored spectrophotometrically by observing culture turbidity at optical density $A_{730}$. The experiment was repeated more than five times using different cell cultures under low and high PFDs.
PSII electron transport activity significantly (Table 3).

We have further tried to reveal photosusceptibility of photosynthetic efficiency (Fv/Fmax) of PSII. The mutant cells showed phototolerance even after high light-induced faster inactivation of PSII activity under photoinhibitory irradiance. In spite of this, mutant cells have always been able to express their photosynthetic potential = 75% of the control cells (Table 2) under normal growth condition. However, declining trend of Fv/Fmax has been shown by both cell types, when placed under strong photoinhibitory irradiance. Mutant cells show a rapid loss of their chlorophyll fluorescence variable yield (Fv/Fmax). Consequently, = 50% loss in photosynthetic efficiency of PSII was monitored within 30-33 min in mutant cells as compared to wild type cells which reached = 50% loss in about two hour under similar light exposure (Fig. 2).

Recovery from photoinhibition

Our results indicate rapidly losing trends of PSII electron transport activity and photosynthetic efficiency by mutant cells as compared to control cells during photoinhibitory irradiance (Fig. 2 and Table 2). To clarify and correlate these differential photosynthetic characteristics with restoration process, both cell types were illuminated (500 μmol m⁻² s⁻¹, 30°C) in such a manner as to create = 50% photoinhibitory loss, and immediately these photoinhibited cells were transferred to recovery under growth light (30 μmol m⁻² s⁻¹, 30°C) with the simultaneous addition of translational inhibitor lincomycin to prevent protein synthesis-dependent repair process of PSII reaction centre (data not shown). In spite of differential photosusceptibility for losing PSII activity and photosynthetic efficiency (Fv/Fmax) both cell types indicated similar intrinsic ability to overcome their photoinhibitory losses (Fig. 3), if allowed to recovery after PI 50%. In fact, the initial one hour of recovery phase has played a very crucial role for repair. In view of this, = 66% of the total restoration occurred within the first hour of recovery duration. Afterwards, it became rather slow and could recover about 60% of the total photoinhibited loss in five hours (Fig. 3).

Darkness did not show any positive role for improving recovery process. However, presence of protein synthesis inhibitor (lincomycin) during recovery induced further loss in the residual chlorophyll fluorescence variables parallel with the incubation time (data not shown).

D1 degradation and resynthesis during recovery

The mutant cells were able to divide even under prolonged high light exposure (Fig. 1B). Surprisingly,
this kind of growth behaviour is just sustained by residual (=20%) electron transport (data not shown) and chlorophyll fluorescence variable (Fig. 2). Mutant cells lost almost 80% of their photosynthetic potential within two hours as compared to control cells, which retained more than 50% PSII activity under similar photo inhibitory conditions. Keeping this in account, D1 photodegradation was also analysed for providing further possibilities associated with D1 turnover linked with photoinhibition (Fig. 4). The levels of D1 photodegradation do not overlap with loss in their photosynthetic efficiency and electron transport. This may be due to an inseparable association of active and inactive forms of D1. Immunoblot analysis corresponds to the sum of active and photodamaged D1 protein at all specific points of photo inhibitory measurements. The wild type cells always showed slower D1 photodegradation for losing electron transport activity and chlorophyll fluorescence yield. However, the half-decay time for D1 was much longer =10 hr and more than 20 hr in mutant and control cells (Fig. 4) as compared to the P1 50% of their chlorophyll fluorescence yield achieved in =30 min and =120 min respectively (Table 2).

Furthermore, D1 photo-degradation and its light and dark dependent re-synthesis patterns were also investigated. A loss in photosynthetic efficiency =50% was chosen as a criteria, as stated earlier. The immunoblots of D1 indicate differential D1 photo-degradation as shown by mutant and control cells. It also indicates that D1 gets re-synthesized sufficiently after photoinhibition, if photoinhibited cells shifted under recovery or growth light (30 μmol m⁻² s⁻¹) for longer duration (5 hr) at optimal growth temperature (30°C). Hence, intensity of D1 signal after recovery under growth light appears higher than the control, indicating nascent D1 synthesis during restoration process as well as its association with residual D1 (damaged and active), and also favouring that D1 translational activities are strictly governed by light. Eventually, the sum of these three forms of D1 makes D1 quantitatively higher in totality under growth light recovery process, as detected immunologically (Fig. 5). However, darkness seems to be uncoupled for any nascent D1 protein synthesis and rather promotes post-illumination degradation of photodamaged form of the D1. This may be due to light-induced protease involvement promoting post-illumination proteolytic degradation of D1 content (Singh et al., data under publication).

**Discussion**

The amino acid sequences of D1 polypeptide between D and E loop have many properties essential for the function and turn-over of PSII under light stress

In view of this, Synechocystis 6803 D1 mutant (16) containing engineered psbAII gene

with three point amino acid substitutions viz., N322I, I326F and F328S in the E-loop of the D1 polypeptide, near C-terminal (Table 1) was analysed. Low and high light growth characteristics of both cell types were observed to evaluate the role of amino acid substitutions, which extended differential photosynthetic properties and phototolerance under normal and photoinhibitory condition (Table 2). Almost an identical growth pattern along with electron transport activity was shown by both cell types under growth light (Fig. 1A, Table 3). However, upon shifting from
Fig. 5—Immunodetection of D1 protein after photoinhibition and after recovery [Immunoblot demonstrating total D1 protein content and its nature of turnover in wild type (KC) and mutant (16) strains of Synechocystis 6803. The cells were grown to the mid-logarithmic phase (A730 = 0.8) under growth light (30 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) prior to photoinhibitory treatment. Lane PI, cells were shifted from growth light to high light (500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) to create ca. 50\% photoinhibition in both types of cells. Lanes LR and DR cells were allowed recover either in growth light or in darkness for 5 hr immediately after photoinhibition. Temperature was maintained 30°C throughout. Cells were harvested at each specific step and subsequently used for thylakoid isolation as stated in ‘Materials and Methods’. Thylakoid membranes were fractionated on SDS-PAGE (16\% with urea 6\% M) on chlorophyll basis (2 \( \mu \text{g} / \text{lane} \)). Resolved polypeptides were transferred onto nitrocellulose membrane and immunologically detected with D1-specific primary antibody followed by anti-rabbit secondary antibody using ECL kit. An increase or decrease in the levels of D1 signals are reflected by environmental variables as indicated].

low to high irradiance (30 to 500 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), 30°C) at an early logarithmic growth phase (A730 = 0.35) wild type cells exhibited an early rising with faster cell multiplication up to 24 hr in the beginning followed by continuous gradual decline, representing typical bell shaped growth pattern (Fig. 1B) due to severe photobleaching. Mutant cells started with slightly slow rise-up in comparison to wild type cells initially, but later showed an increase in their growth trend (S-shape) and also retained blue-green appearance throughout. This indicates that wild type cells suffered photobleaching (both phyocobilisomes and chl a ), which impaired processing of sufficient photosynthetic to keep cells photosynthetically dynamic as seen in their absorbance spectra (data not shown). The PBS degradation might have contributed to a small extent for cell survival in the beginning of light stress in KC cells, and it also appears to be critical with mutant cells for conferring their survival and phototolerance under high PFDs. The reduced PBS levels become more critical when cells experience higher light intensities or exposed to the combination of stresses\(^7\). Furthermore, phototolerant mutant cells were also analysed for PSI electron transport rates, chlorophyll fluorescence variables and photodegradation of D1 to correlate physiological basis favouring high light adaptation. Surprisingly, an early loss in PSI \( O_2 \) evolving capacity, \( Fv/Fmax \) and D1 photodegradation were found in mutant cells whereas, wild type cells indicated rather slower way for losing all these intrinsic properties. Noticeably, \( \approx 50\% \) loss in photosynthetic efficiency occurred within 30-33 min in mutant cells while, it takes almost 120-130 min in wild type cells (Table 2). To the best of our knowledge, it is the first \( \text{psbA} \) mutant reported so far showing typical nature of photo-tolerance\(^\text{27} \) with an early loss in their PSI electron transport (H\(_2\)O-DCBQ), photosynthetic efficiency, and D1 photodegradation in comparison to the wild type cells. Eventually, mutant cells lost \( \approx 80\% \) PSI electron transport (data not shown) and \( Fv/Fmax \) values within two hours of continuous photoinhibitory illumination (Fig. 2). In spite of this, mutant cells sustained their growth potential by retaining normal phenotypic appearance along with photosynthetic pigments, and spectral properties (data not shown). This may be interpreted as that wild type cells kept on losing all these intrinsic characteristics gradually till the end (Fig. 1B) while, mutant cells chose biphasic strategies. A rapid loss followed by steady state around \( \approx 20\% \) residual photosynthetic efficiency could support cell growth sufficiently under strong irradiance with lower photosynthetic ability\(^\text{17,28} \). Oquist \textit{et al.}\(^\text{29} \) have also suggested that photoinhibited PSI reaction centres (with photodamaged D1 protein) as they accumulate, extend an increased photo-protection for the remaining functional PSI by controlled non-photochemical dissipation of excess excitation energy to establish down-regulation of PSI under sustained strong PFDs, which regulate resistance against irreversible photodamage\(^\text{30} \) in shade and low light grown plants.

So far no report exists to our knowledge stating such a kind of phototolerance behaviour in Synechocystis. However, reports do exist on amino acid substitutions in D1 that favour either more or equally photosusceptible D1 mutants\(^\text{31,32} \). Whereas, a conditional i.e., higher or equal levels of phototolerance
has also been observed in Synechocystis\footnote{9}. The loss in photosynthetic efficiency (Fv/Fmax) under photo-inhibitory irradiance has been correlated with the inhibition of PSII electron transport due to donor or acceptor side limitation or perturbation\footnote{4,24}. In our experiments, because of three point amino acid substitutions, mutant cells reflected a 50% photosynthetic oxygen evolving capacity under normal growth condition, when assayed using DDBQ as an electron acceptor (Table 3) as compared to the wild type. However, PI 50% duration of electron transport activity was slightly higher with the similar pattern to the chlorophyll fluorescence values (Table 2) as monitored with DDCBQ, which accepts electrons directly from QA\footnote{46,49}.

Apart from D1 mutational consequences, we could not get specific clue of phototolerance as shown by mutant 16 cells based on their sustainability towards cell multiplication, ability for retaining photosynthetic pigments, rapid loss in electron transport and chlorophyll fluorescence variables. Generally, D1 mutants represent modified acceptor side of PSII, including herbicide-resistant mutants\footnote{40,47,48}, the pshH deletion mutant\footnote{49,50}, the PEST mutant\footnote{51} and all have shown intrinsically either equal or higher susceptibility towards photoinhibitory damage of PSII than the wild type. However, restoration of photo-inhibited photosynthetic efficiency was observed more rapidly under normal growth light after photo-inhibition (Fig. 3), while darkness did not favour restoration (data not shown). The rapid first phase of recovery occurred within the first hour of the restoration process, and contributed to 66% of the total recovery achieved in five hours. The light dependent recovery indicates strict translational regulation of D1, and accumulation or stabilization of full length D1 protein\footnote{52,44}, although the exact mode of regulation is not known.

Furthermore, loss in photosynthetic efficiency (Fv/Fmax), and PSII photoactivation were accompanied with slow D1 photo-degradation process in both cell types. However, mutant cells showed faster D1 photo-degradation with the half-decay time =10 hr than the wild type cells having >20 hr, which could be due to possible conformational changes after amino acid substitutions (Table 1, Fig. 4). In contrast to this, slower D1 degradation in mutants was observed under strong irradiance\footnote{53}. However, light dependent recovery process focused a better D1 signal, as detected by using specific D1 antibody, possibly due to an integrated immunodetection of D1 which includes nascent, damaged, and already existing active forms of D1\footnote{54,45} while, defective synthesis or stabilization of the D1 protein has also been thought to be an underlying reason for the failure to repair damaged PSII reaction centres during darkness (Fig. 5).

The light induced inhibition of photosynthetic efficiency of PSII also generates protease, which renders D1 degradation in vivo and in vitro during post-illumination under darkness\footnote{55,56} (Singh and Satoh, data under publication). Further studies to explore a better insight regulating phototolerance with sustainable cell growth and maintenance endowed with unaffected spectral properties along with residual (~20%) PSII efficiency under prolonged photo-inhibitory irradiance as opted by phototolerant mutant are warranted.

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References
