Site-specific mutations localized in the D-E loop of the D1 protein of photosystem II affect phototolerance in *Synechocystis* sp. PCC 6803 containing *psbAII* gene

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Photosynthetic characteristics along with phototolerance and photoinhibition of photosystem II (PS II) were monitored in *Synechocystis* sp. PCC 6803 wild type (KC) and its *psbAII* mutants viz., I6 (N322I, I326F, and F328S), G6 (N267Y), and H7 (Y254C and I314V) that have up to three point mutations, localized in the D-E loop of the D1 polypeptide of PSII reaction centre. These strains exhibited entirely different growth trends upon shifting from 30 $\mu$mol m$^{-2}$s$^{-1}$ to high irradiance (500 $\mu$mol m$^{-2}$s$^{-1}$, 30°C). The I6 and H7 cells grew well, whereas KC and G6 cells showed inability for cell multiplication. The photosynthetic efficiency demonstrated about 50% loss in chlorophyll fluorescence of variable yield (Fv/Fm) within 20-30 min in all mutants, whereas the wild type (KC) cells could reach the same level of loss in 2 hr. I6 and H7 cells showed continuous cell growth and maintenance under long-term exposure of high light compared to G6 mutant and wild type cells. The wild type cells showed slow decrease in their photochemical activity and Fv/Fm values, compared to mutant cells. The recovery seemed to be almost identical, and also stimulated by growth light, inspite of differential photoinhibitory behaviours. Darkness and translational inhibitor lincomycin both were found to be unassociated with the restoration of photoinhibited process of PS II.

The D1 protein of PS II is more light sensitive compared to other photosynthetic reaction centre core proteins. It possesses an inherent rapid turn-over tendency which sustains photosynthetic efficiency under photoinhibitory and non-photoinhibitory irradiance$^{1,2}$. The photosynthetic organisms need light for their photoautotrophic growth, but they may also experience loss in their photosynthetic oxygen evolution or CO$_2$ assimilation, if placed under high light intensity coupled with either elevated or low temperature$^{3,4}$. Generally, it is accepted that the target of such kind of light-dependent damage lies in the D1 reaction centre core protein of the PSII$^{10,11}$, having five trans-membrane helices$^{12}$, in which primary cleavage occurs in the D-E loop during light stress$^{13}$. Thus, the D-E loop is of utmost significance for PSII functioning$^{14}$ under strong irradiance. It also provides binding niche for the two electron accepting species of quinone known as Q$_B$$^{15}$, which is associated with photosensitivity$^{10,16}$.

Mutagenesis technique has been employed in cyanobacteria to reveal structural and functional relationship of various amino acids localized in the D-E loop of the D1 polypeptide$^{14,17}$. Since D1 mutational consequences could alter phototolerance$^{18,19}$, therefore, cyanobacterium *Synechosystis* sp. PCC6803 and its D1 mutants viz., I6, G6 and H7 (created by Narusaka *et al.* 1999, ref. 17) were chosen to elucidate the mechanism of phototolerance. The results indicate that the particular combination of amino acids in the D-E loop of the D1 protein affects functional characteristics of PSII$^{18}$. Consequently, I6 (Asn322Ile, Ile326Phe and Phe 328Ser) and H7 (Tyr254Cys and Ile314Val) mutants have shown phototolerance, inspite of their reduced chlorophyll fluorescence of variable yield (Fv/Fm) under high irradiance$^{18}$. However, wild type (WT) and mutant G6 (Asn267Tyr) cells showed photosusceptibility under similar high light growth condition. The chlorophyll fluorescence reflects the regulation of absorbed light energy dissipation as well as its utilization$^{20}$. The absorption of light often exceeds the energy utilization capacity of PSII, therefore, the regulation of excess light energy through phosphorylation and

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dephosphorylation of light harvesting complex II promotes balanced energy transfer to the LHC I complex to avoid the severity of photoinhibition of PS II in higher plants\(^8,21,22\). Our findings indicate phototolerance shown by I6 and H7 mutant cell types under long-term high light exposure with residual chlorophyll fluorescence variable yield (Fv/Fm).

**Material and Methods**

*Experimental organism and culture conditions*

Cyanobacterium *Synechocystis* PCC 6803 having multiple copies of *psbA* (*psbAI, psbAII* and *psbAIII*) gene\(^23\) with *psbAI* and *psbAIII* gene forms inactivated was chosen as an experimental organism. Various *psbAII* mutants from wild type (KC) strain were generated by random PCR\(^17\). Mutants I6, H7 and G6 have shown three (N322I, I326F, F328S), two (Y254C, I314V) and one (N267Y) point amino acid (s) substitutions, as confirmed by *psbAII* gene sequencing (Fig. 1), based on their single colony PCR\(^18\). The wild type and its *psbAII* mutants were grown in BG11 liquid cultures\(^24\) containing antibiotics kanamycin (5 \(\mu\)g/ml), spectinomycin (5 \(\mu\)g/ml) and chloramphenicol (2.5 \(\mu\)g/ml), while the host strain (KC) and its *psbAII* mutants were grown in liquid BG11 at 30°C with continuous bubbling by using sterile atmospheric air containing 1% CO\(_2\) supply for efficient mixing of the growing cells. The cell cultures were illuminated with growth photon flux densities (PFDs), using white fluorescent bulb (30 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\)), unless mentioned otherwise. The cells grown to mid-logarithmic growth phase (A\(_{730}\) ~0.8) were used for all studies.

*Cell growth and chlorophyll measurements*

The cell growth was measured through monitoring cell-turbidity spectrophotometrically (Milton-Roy, Spectronic 3000) at A\(_{730}\)nm in a cuvette with 1 cm light path\(^24\). Chlorophyll contents of the cells and thylakoids were estimated by using 80% acetone\(^25\).

*In vivo photoinhibitory (PI) treatments and chlorophyll fluorescence measurements*

The cells grown to mid-logarithmic growth phase (A\(_{730}\)nm ~0.8) in BG11 medium were photoinhibited under high PFDs ( 500 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) at 30°C). The measurements of Fv/Fm from photoinhibited *Synechocystis* cells were made by using PEA (Plant efficiency analyzer, Hansatech, UK). For recovery, the cells were photoinhibited to about 50% of the Fv/Fm (variable fluorescence per maximum fluorescence) value at the beginning\(^26\). This was accomplished in about 30 and 120 min with mutants and WT cells, respectively. The cell suspension (2 ml) in a cuvette was used at specific time period for all measurements containing 10 \(\mu\)g Chl after keeping in darkness at 30°C for 15 min, which allowed relaxation of fast fluorescence-quenching components. The translational inhibitor, lincomycin (400 \(\mu\)g/ml, Sigma) was added immediately after photoinhibition treatment, prior to transferring the cells for recovery process\(^14\).

*Preparation of thylakoid membranes and Western blot analysis*

Cyanobacterial cells grown up to late growth phase (A\(_{730}\)nm ≥1.5) were harvested (10,000 g, 10 min, 4°C) and the thylakoids were isolated for Western blot analysis\(^2,18\). The polypeptides of the thylakoid membranes equivalent to 2 \(\mu\)g of Chl were fractionated by SDS-polyacrylamide gel (16%) containing 6 \(M\) urea. The fractionated polypeptides were then transferred onto 0.2 \(\mu\)m nitrocellulose membrane\(^2,18\). The blots were probed with primary D1 and D2
protein-specific antibodies (kindly gifted by Prof. Ikeuchi, Tokyo University, Tokyo, Japan) followed by secondary anti-rabbit antibody (Amersham), and subsequently treated with chemiluminescence kit (ECL, Amersham) to obtain the signals on the X-ray film (Fujifilm, Japan).

Results

Growth characteristics under high PFDs

The phenotypic appearances of the mutants i.e. I6, H7 and G6 having triple (N322I, I326F, F328S), double (Y254C, I314V) and single (N267Y) point mutation(s) in their D1 polypeptide (Fig. 1), respectively and wild type cells were almost identical under low growth irradiance at 30°C (data not shown). However, all the cell types exhibited entirely different growth responses, upon shifting at their early log growth phase (A730nm≈0.35) to photoinhibitory irradiance (500 μmol m⁻² s⁻¹, 30°C). WT cells showed faster growth up to 30 hr, but afterwards they failed to grow (Fig. 2), and also exhibited photobleaching of the photosynthetic pigments i.e., phycobilisome (PBS) and Chl a (data not shown). On the contrary, I6 and H7 mutants showed slow rise in the beginning (up to 30 hr), but later sustained their cell maintenance and multiplication with typical S-shape growth curve (Fig. 2). However, G6 mutant cells failed to grow under similar growth condition (Fig. 2). The D1 mutational sites as shown in Fig. 1 did seem to affect photosensitivity and photosynthetic efficiency (Fv/Fm) of PSII in all mutants, compared to WT (KC) cells (Table 1), as WT cells showed higher chlorophyll fluorescence variable yield. They grew well under low growth light compared to all mutant cells (Table 1).

Chlorophyll fluorescence variable yield (Fv/Fm) of PSII under photoinhibitory PFDs

The observations were also made to find out the high light (500 μmol m⁻² s⁻¹, 30°C) responses of various D1 mutants. The 50% loss in Fv/Fm value was observed quickly (20-33 min) in I6, G6 and H7 mutant cells, while WT cells took nearly four times longer duration (Table 1 and Fig. 3). All the three mutant cells demonstrated biphasic loss in Fv/Fm i.e., initially rapid, followed by slow phase. Though, G6 and H7 mutant cells showed severe photoinhibition with almost 75% loss in their Fv/Fm in 1 hr, I6 and control (WT) cells could reach the photoinhibitory loss of about 55% and 30%, respectively under similar high light exposure (Fig. 3).

Restoration of photoinhibited chlorophyll fluorescence variable yield (Fv/Fm)

The 50% loss in Fv/Fm value was attained by WT and mutant cells differentially (Table 1). The mutant

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fv/Fm % control</th>
<th>Time taken for loss in 50% of the initial value</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Fv/Fm (min)</td>
</tr>
<tr>
<td>KC</td>
<td>100(0.550)</td>
<td>120-130</td>
</tr>
<tr>
<td>I6</td>
<td>73(0.400)</td>
<td>30-33</td>
</tr>
<tr>
<td>G6</td>
<td>85(0.470)</td>
<td>20-22</td>
</tr>
<tr>
<td>H7</td>
<td>85(0.466)</td>
<td>20-22</td>
</tr>
</tbody>
</table>

Table 1—Photosynthetic characteristics of cyanobacterium Synechocystis 6803, wild type (KC), and its psbAII mutants (I6, G6 and H7) cells

[Values in parenthesis are the mean values (5-7 independent experiments) of the Fv/Fm observed at normal growth irradiance. Cells were illuminated continuously under photoinhibitory light (500 μmol m⁻² s⁻¹) to create nearly same level of photoinhibition (PI) ~50% after achieving their logarithmic growth phase (A730 =~0.8) under growth light (30 μmol m⁻² s⁻¹). The temperature was maintained 30°C throughout]
I6 showed 50% loss in ~30 min, whereas the G6 and H7 mutants could reach the same level of photoinhibitory loss in 20 min. However, WT cells exhibited slow loss in their PSII photosynthetic efficiency, and 50% loss occurred at almost four times longer duration of ~120 min. Inspite of differential photoinhibitory loss in their variable chlorophyll fluorescence yield under identical photoinhibitory irradiance, all cell types exhibited almost identical extent of recovery, if kept under low growth light after attaining 50% loss in their Fv/Fm (Figs 4A, B, C and D). Almost 60% of the total recovery occurred within first hr of the fast phase of the recovery process. The recovery process was arrested by translational inhibitor, lincomycin that inhibited protein synthesis-dependent repair process in all cell types (Fig. 4A, B, C and D). Lincomycin affected less in the restoration of photoinhibited photosynthesis in WT cells at the beginning, but later showed similar trend as found with the mutant cells (Fig. 4A).

Western blot analysis of D1 and D2 reaction centre core proteins

Since all mutants have shown variability in their growth and photosynthetic efficiency of PS II as monitored by Fv/Fm values under normal (Table 1) and strong light (Fig. 2), D1 and D2 core proteins of the PS II were also analyzed immunologically by using specific antibodies to estimate their steady state levels. We found that the mutagenesis of the D-E loop of the D1 polypeptide affected not only D1 protein, but also impaired D2 protein of PS II as well (Fig. 5) in all mutant cell types, compared to WT cells. The I6 cells were found more affected than the G6 and H7...
The D-E loop of the D1 polypeptide has many essential properties linked with the functioning of PSII under light stress\(^1\). In view of this, combination of amino acids could be expected to regulate the photosynthetic efficiency and phototolerance\(^2\). Therefore, amino acid substitutions as indicated in Fig.1 seem to be involved in modifying the conformational changes of the D1 polypeptide in all mutants. Consequently, all mutant cells responded differentially in their photoautotrophic growth under high PFDs (Fig. 2), and also PSII characteristics to maintain Fv/Fm under low growth light (Table 1). The mutational consequences favoured conformational changes in the D1 polypeptides of all mutants. Since H7 and I6 mutants which represent two and three point amino acid substitutions were found phototolerant compared to photosensitive G6 mutant having only single point mutation, it appears that overall two or more number of specific amino acid substitutions viz., Tyr254Cys, Ile314Val, and Asn 322 Ile, Ile 326 Phe, Phe 328 Ser (Fig. 1) induced phototolerance in H7 and I6 mutants with the ability of cell growth (Fig. 2). However, G6 mutant showed strong photosusceptibility, and also failed to grow possibly because of single point mutation i.e., Asn254Tyr (Fig. 1) under high light (Fig. 2).

The H7 and I6 mutant cells could retain their spectral properties, whereas WT cells failed to do so by decreasing their phycobilisome (A\(_{620nm}\)) peak height (data not shown). WT cells also faced retardation in their cell multiplication (Fig. 2). Therefore, photobleaching seems to be integrated with cell growth as shown by WT and G6 mutant cells. Phototolerance linked to PSII in photosynthetic organisms mainly occurs due to an efficient energy dissipation or leakage at the stage of excitation energy transfer in the pigment system\(^26,28\).

The I6 and H7 phototolerant mutants adapted an unique way for expressing the phototolerance coupled with their cell multiplication (Fig. 2), and avoidance of photobleaching (data not shown) with quick loss in chlorophyll fluorescence variable yield (Fig. 3). A rapid loss in photosynthetic efficiency of PSII, followed by steady state residual Fv/Fm in H7 and I6 mutants might have extended an ability for continuous cell growth (Fig. 2) with low photosynthetic efficiency. Possibly, a small fraction of PS II reaction centres remain functional under irradiance stress which sustain electron flow from H\(_2\)O to ferredoxin at rates sufficient for photosynthesis and cell growth\(^29\). The photoinhibited PSII reaction centres (with photodamaged D1 protein) as they accumulate, confer increased photo-protection to the remaining functional PSII centres by controlled non-photochemical dissipation of excess excitation energy with down-regulation of PSII under sustained high irradiance to regulate resistance against irreversible photodamage\(^30\). Under conditions of occasional high-light exposure, the PSII centres may become easily photoinhibited, because of concurrent slow recovery process. However, photoinhibited PSII reaction centres remain structurally intact in the apprised parts of the thylakoid membranes, still efficient in trapping the excitation energy, but dissipate it non-photochemically\(^31\). Consequently, it appears that *Synechocystis* phototolerant mutants I6 and H7 cells grown in low light managed their cell growth without maintaining an energetically expensive and fast repair of the PSII\(^32\) under sustained photoinhibitory PFDs. However, WT cells lost their spectral properties and cell-multiplication ability at high PFD (Fig. 2). This has ultimately placed WT cells under photosusceptible category. The restoration of photo-inhibited efficiency (Fv/Fm) in WT and its mutant cells seems to be totally dependent on protein synthesis (Fig. 4 A, B, C and D) process\(^33\).
The herbicide-resistant mutants with modified acceptor side of PSII carrying point mutations in the Q_b binding site\textsuperscript{34} and strains with point mutations or small deletions in the PEST (225-238 amino acid sequences of the D1 protein) and cleavage-site regions have been reported to be intrinsically either equally or more sensitive to light induced damage of PSII than wild type\textsuperscript{35}. The phototolerance was observed in an autotrophic D1 mutant (ΔR225-F239, PD) of Synechocystis PCC 6803, when oxygen evolution was monitored with dichlorobenzoquinone (DCBQ), but found to be equally susceptible compared to WT cells when monitored using bicarbonate, suggested an inactivation of Q_b binding niche as the first event in the photoinhibition cascade in vivo\textsuperscript{14}. The restoration of photoinhibited Fv/Fm in WT and its mutant cells seemed to be totally dependent on protein synthesis (Fig. 4 A, B, C and D) process\textsuperscript{37}. It is also conceivable that under growth-light mutated and misfolded D1 reaction centre core polypeptides have effectively been degraded by non-specific household protease\textsuperscript{14}, which cause lowering in the D1 content in all mutants (Fig. 5). The D1 mutational consequences impaired D2 content in all mutant cell types (Fig. 5), due to transmembrane effect\textsuperscript{14}. This indicates the existence of co-interacting factors regulating synthesis of D1 and D2 in association, but not in isolation. So far, no report exists to the best of our knowledge\textsuperscript{18} favouring such kind of phototolerance in cyanobacteirum Synechosystis as expressed by I6 and H7 mutants. Thus, further experiments are required to find out specific clue of phototolerance based on an ability to maintain cell growth and photosynthetic pigments or avoidance of photobleaching with quick loss of photosynthetic efficiency of PSII along with lower D1 and D2 protein contents as shown by I6 and H7 phototolerant mutants containing engineered psbAII gene.

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