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Parallel expression of alternate forms of *psbA2* gene provides evidence for the existence of a targeted D1 repair mechanism in *Synechocystis* sp. PCC 6803☆

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ABSTRACT

The D1 protein of Photosystem II (PSII) is recognized as the main target of photoinhibitory damage and exhibits a high turnover rate due to its degradation and replacement during the PSII repair cycle. Damaged D1 is replaced by newly synthesized D1 and, although reasonable, there is no direct evidence for selective replacement of damaged D1. Instead, it remains possible that increased turnover of D1 subunits occurs in a non-selective manner due for example, to a general up-regulation of proteolytic activity triggered during damaging environmental conditions, such as high light. To determine if D1 degradation is targeted to damaged D1 or generalized to all D1, we developed a genetic system involving simultaneous dual expression of wild type and mutant versions of D1 protein. Dual D1 strains (nS345P:eWT and nD170A:eWT) expressed a wild type (WT) D1 from ectopic and a damage prone mutant (D1-S345P, D1-D170A) from native locus on the chromosome. Characterization of strains showed that all dual D1 strains restore WT like phenotype with high PSII activity. Higher PSII activity indicates increased population of PSII reaction centers with WT D1. Analysis of steady state levels of D1 in nS345P:eWT by immunoblot showed an accumulation of WT D1 only. But, in vivo pulse labeling confirmed the synthesis of both S345P (exists as iD1) and WT D1 in the dual strain. Expression of nS345P:eWT in FtsH2 knockout background showed accumulation of both iD1 and D1 proteins. This demonstrates that dual D1 strains express both forms of D1, yet only damage prone PSII complexes are selected for repair providing evidence that the D1 degradation process is targeted towards damaged PSII complexes. Since the N-terminus has been previously shown to be important for the degradation of damaged D1, the possibility that the highly conserved cysteine 18 residue situated in the N-terminal domain of D1 is involved in the targeted repair process was tested by examining site directed mutants of this and the other cysteines of the D1 protein. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

1. Introduction

Susceptibility of PSII to photoinhibition depends upon the balance between photodamage and repair (recently reviewed in Refs. [1,2]). Therefore, to maintain higher PSII activity in a cell there is a continuous flux between damage and repair of PSII. This damage and repair cycle can be conceptually divided as the damage causing PSII to be inactivated, sensing the damage to initiate repair and the actual process of repair to form active PSII. Studies on PSII repair have shown that D1 protein is the main target for photodamage and this was noticed with the increased turnover rate of D1 in comparison to other PSII proteins during photoinhibition. In vivo pulse chase experiments showed the presence of a repair process wherein D1 undergoes degradation and synthesis [3–9]. The synchronous nature of D1 degradation and synthesis was also demonstrated using translation inhibitors [10]. Taken together, from these studies and others, it is reasonable to assume that PSII repair specifically replaces the damaged D1 subunits with newly synthesized D1. Based on recent findings, it is known that the exposure of the N-terminus is required for recognition and proteolytic attack by FtsH2 [7,11]. Accordingly, it seems reasonable to suppose that damaged D1 proteins undergo a damage-triggered conformational change resulting in this exposure as part of a molecular signal, 'flagging' PSII

Abbreviations: Chl, chlorophyll; D1, reaction center protein encoded by the *psbA* gene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Em^R, erythromycin resistance cassette; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HBG-11, BG-11 growth medium buffered with Hepes–NaOH pH 8; Km^R, kanamycin resistance cassette; MES, 2-(*n*-morpholino) ethanesulfonic acid; Mn_4CaO_5, metal cluster functioning in H_2O oxidation; PAGE, polyacrylamide gel electrophoresis; PSII, Photosystem II; P_{680}, primary electron donor in PSII; PVDF, poly vinylidene difluoride; Q_A, PSII primary electron acceptor strongly influencing fluorescence yield; Q_B, the secondary quinone acceptor of PSII; Y_z, redox active tyrosine of the D1 protein acting as a secondary electron donor of the reaction center

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complexes for the initiation of repair involving replacement with a new copy of the D1 protein.

However, prior to addressing the question of the molecular nature of the hypothesized damage signal, it is pertinent to first address the question: Are only damaged proteins targeted for PSII repair? Or does photoinhibition trigger the replacement of all D1 subunits by up-regulation D1 turnover rate in a generalized manner? To address this question, it is critical to visualize that during photoinhibition only a subset of PSII complexes from the entire pool of PSII existing in a cell incurs photodamage. Hence, the increased turnover rates of D1 observed from pulse-chase protein labeling experiments do not provide unequivocal explanation on the type of D1 subunit, (damaged or both damaged and undamaged) being replaced. Therefore, both the alternatives of targeted and generalized PSII repair are indistinguishable considering the available indirect evidence in the form of; for example, pulse-labeling studies, which cannot identify damaged protein per se, and determines only the net proteolytic turnover of D1 protein.

In this study we have developed a genetic system to express two alternate forms of D1 to determine if cells can distinguish between damaged and undamaged D1 during the repair process. The genetic system was developed using a D1 ectopic strain constructed previously [12] as a background recipient *Synechocystis* sp. PCC 6803 (*Synechocystis*) strain into which a second copy of D1 was expressed. In *Synechocystis*, D1 protein is encoded by three genes *psbA1*, *psbA2* and *psbA3* that are differentially expressed under different conditions in a light dependent manner with *psbA2* contributing to 95% of transcript levels [13–15]. *PsbA2* and *psbA3* share a 99% nucleotide identity and encode for an identical gene product, yet *psbA2* is the highly expressed *psbA2* gene in *Synechocystis* [16]. Therefore, we primarily targeted *psbA2* for construction of the genetic system.

The principle behind the development of this system as illustrated in Fig. 1, was to allow parallel expression of wild type (WT) and damage prone D1 simultaneously in a cell thereby forming two populations of PSII complexes. If the damage prone D1 is specifically targeted for repair then the increased turnover rate of damaged D1 will essentially result in the accumulation of WT D1 protein capable of forming active PSII reaction centers (RCs). On the other hand, if there were a generalized repair mechanism then both the WT and damage prone D1 copies would have an increased rate of turnover leading to an equal incorporation of both forms of D1 in PSII RCs accumulating similar proportions of active and inactive PSII (Fig. 1).

2. Material and methods

2.1. Strains used in this study

Constructs used in this study can be categorized as control, single, and double *psbA2* mutant strains all constructed using a triple *psbA* deletion background (*ΔA1*:*ΔA2Em*:*ΔA3*) in the naturally transformable, glucose-utilizing strain of *Synechocystis* grown in shaking culture at a light intensity of ~30 μmol m^{-2} s^{-1} incident [12,14]. Strains were constructed by re-introducing one or two different *psbA2* alleles into the triple *psbA* deletion background. The control strains nWT and eWT express wild type *psbA2* gene from the native (n) and ectopic (e) loci on the chromosome. Single *psbA2* mutant strains n-S345P, n-D170A and n-H337Y express the mutant forms of D1 having mutations D1-Ser345Pro [11,17,18], D1-Asp170Ala [18,19] and D1-His337Tyr [20] from the native *psbA2* locus. These mutations were selected based on previously reported characteristics. D1-Ser345Pro (D1-S345P) mutation has been shown previously to prevent carboxy terminus processing of D1 causing an inherent instability of the protein [11,17,18]. D1-Asp170Ala (D1-D170A) mutation prevents assembly of the manganese cluster and has been shown to have a higher D1 turnover rate as well [18,19]. The D1-His337Tyr (D1-H337Y) mutant has been shown to compromise the ability to accumulate functional PSII due to light-sensitive phenotype [20]. All three mutations result in lower accumulation of PSII complexes when compared to WT. The construction of the double *psbA2* mutant strains nS345P:eWT, nD170A:eWT and nH337Y:eWT is described in Subsection 2.2 and involves the expression of mutant *psbA2* in parallel with the eWT strain. FtsH2 (encoded by *slr0228*) knockouts were also constructed for nS345P:eWT by replacing the *slr0228* with a spectinomycin resistance cassette.

Additionally, D1-C18A and D1-Cysless strains were constructed to study the role of conserved D1 cysteines in PSII repair (Table 1). D1-C18A incorporated a point mutation Cys18Ala generated using site directed mutagenesis of WT *psbA2*. Similarly, the D1-Cysless strain was constructed by replacement of four native cysteines from WT *psbA2*

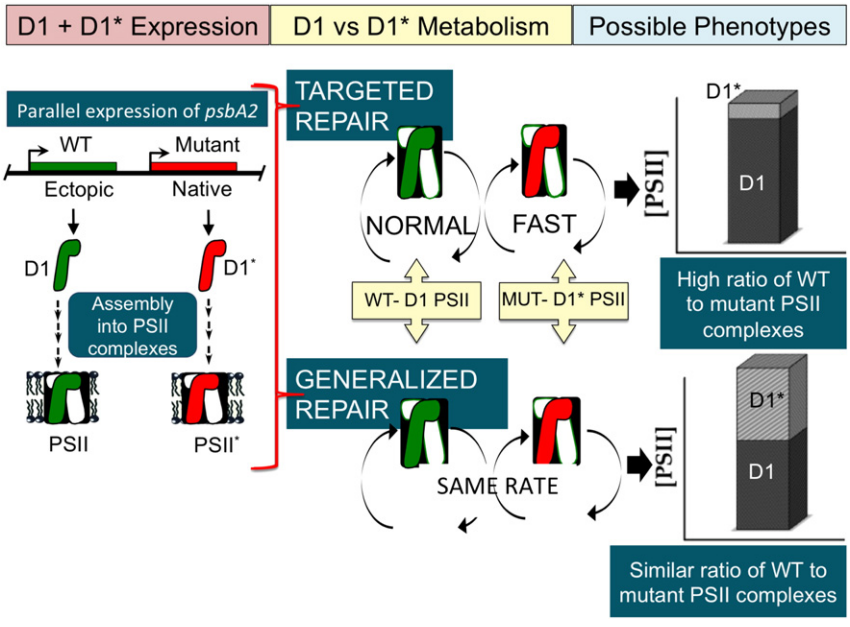

Fig. 1. Schematic representation of the strategy to distinguish between targeted and generalized PSII repair hypotheses. Parallel expression will involve simultaneous WT and mutant (D1 & D1*) expression with the mutant D1* being damage prone and highly turned-over. Based on the mechanism of PSII repair occurring in the cell the rates of D1 turnover would differ. The predicted phenotypes (right) would represent ratio of WT and mutant accumulated in PSII RCs.

Table 1
List of strains used in this study.

Strain	Genotype		Comments
	Native	Ectopic	
n-WT	WT *psbA2* with Km^R	Empty	*ΔA1*:*ΔA2Em*:*ΔA3* used as background recipient strain
e-WT	*psbA2* replaced by Em^R	WT-*psbA2*	Constructed in a previous study, described as *psbA2*NS
D1-S345P	D1-S345P *psbA2* with Km^R	Empty	C-terminal high turnover point mutation in D1
D1-D170A	D1-D170A *psbA2* with Km^R	Empty	Donor side point mutation affecting Mn_4CaO_5 assembly
D1-H337Y	D1-H337Y *psbA2* with Km^R	Empty	Point mutation causing increased susceptibility to photoinhibition
nS345P:eWT	D1-S345P with Km^R	WT-*psbA2*	Parallel expression of WT and mutant *psbA2*
nD170A:eWT	D1-D170A with Km^R	WT-*psbA2*	Parallel expression of WT and mutant *psbA2*
nH337Y:eWT	D1-H337Y with Km^R	WT-*psbA2*	Parallel expression of WT and mutant *psbA2*
ΔFtsH-nS345P:eWT	D1-S345P with Km^R	WT-*psbA2*	Replacement of *slr0228* with Spectinomycin resistance marker
D1-C18A	Cys18Ala *psbA2* with Km^R	Empty	Site directed point mutant in triple *psbA* deletion background
D1-Cysless	D1-Cysless *psbA2* with Km	Empty	Four native Cys 18, 47, 125 and 144 were replaced with Ala

(C18, C47, C125 and C144) with alanine. Table 1 lists all the strains used in the study with their genotype and brief descriptions.

2.2. Construction of dual psbA2 expression strains

Dual *psbA2* strains were constructed by using the WT ectopic strain as a background. The construction of this ectopic strain (*psbA2*NS) has been previously described in Ref. [12]. The location for the ectopic integration of WT *psbA2* has been previously used to probe recombination characteristics and construction of promoter probe vectors [14,21]. The ectopic strain will henceforth be designated as eWT (ectopic WT) to distinguish between the native (n) and ectopic (e) loci on the chromosome. Dual expression strains always carried the WT *psbA2* at the ectopic location and the mutant *psbA2* (damage/turnover prone) at the native location. This was chosen to prevent any gene location defects that could alter mutant phenotype. Additionally, WT *psbA2* gene at the ectopic location is under the control of native *psbA2* promoter, 3′ and 5′ UTR, conferring similar gene regulation at both loci. The expression of ectopic *psbA2* has been confirmed previously to have a function identical to the native counterpart [12].

Mutant *psbA2* genes were introduced into the native locus on the chromosome by transformation of eWT with DNA isolated from previously described high D1 turnover mutants [11,17–19]. This approach allows re-examination of well-studied D1-mutations in conjunction with a WT *psbA2* expressed in parallel. The *psbA2* gene at the native locus bears a kanamycin resistance (Km^R) gene cassette downstream of the gene. Genomic DNA from existing *psbA2* mutants (S345P, D170A, and H337Y [18,20,22]) were isolated and the *psbA2* locus with the downstream Km^R cassette from each mutant genome was PCR amplified using primers P1 (GTCGGAGTTTCGCCTCAAGAT) and P2 (CAGATGTCGTTGCTGGTTACA). The resultant 3900 bp PCR amplified product for each mutant was directly used for transformation into the eWT strain of *Synechocystis*. The transformants obtained were selected for kanamycin resistance.

Integration of mutant at the native and WT *psbA2* at the ectopic loci was confirmed by PCR amplification of the entire native and ectopic site for each strain. Table 2 lists PCR primers used for the confirmation of the *psbA2* gene at both loci. PCR products were sequenced to ensure incorporation of the desired point mutation as well as to confirm that no other random mutations were created during the amplification process (Fig. 2 gel image showing gene integration). Cultures were maintained in liquid BG-11 media in the presence of 5 mM glucose (photoheterotrophic growth) to neutralize the selective advantage for possible revertants. The native and ectopic loci of the two strains were periodically sequenced to monitor any possible recombination event between the two copies of *psbA2* gene.

2.3. PSII activity measured using chlorophyll variable fluorescence

Fluorescence measurements were performed with a double-modulation kinetic chlorophyll fluorometer fitted with a second actinic flash illumination source (PSI Instruments, Brno, Czech Republic). Cells from the mid-exponential growth phase were harvested, resuspended in fresh BG-11 to a density corresponding to 100 μg of Chl/ml and kept under dim light (~25 μmol m^{-2} s^{-1}) with constant shaking. Samples were then diluted to 5 μg of Chl/ml prior to measurements. Variable fluorescence was measured as described previously [23]. The low fluorescence F_0 state in dark-adapted samples was measured by probing fluorescence yield with four measuring pulses followed 200 μs later by a 30 μs saturating actinic flash, followed by a sequence of measuring pulses beginning 50 μs after the actinic flash. PSII has a high fluorescent yield when it is in the $P680Q_A^-$ state, which is formed when P680 absorbs a photon of light and donates an electron to Q_A. Without inhibitors, the principal component of the decay of this state is due to the oxidation of Q_A^- by a plastoquinone in the Q_B site. When DCMU blocks the transfer of electrons from Q_A to Q_B, this causes $P680Q_A^-$ to persist until the electron recombines with oxidants on the donor side.

Since this study uses mutants primarily defective in the donor side of PSII, the decay of fluorescence in the presence of DCMU was further analyzed by normalizing the amplitudes of variable fluorescence for each strain. In the presence of an intact Mn_4CaO_5 when the forward electron flow to Q_B is blocked (by DCMU), the only option for the electron from Q_A^- is to find its way back to Mn_4CaO_5 and this is a much slower process. In the absence of an intact functional Mn_4CaO_5 there is a rapid recombination between the Q_A^- and Y_Z^+ as the Mn_4CaO_5 is not available for the reduction of Y_Z^+. This was useful in determining any heterogeneity in the PSII population due the expression of alternate copies of *psbA2*.

Table 2
Primer pairs used for the amplification of the entire ectopic site (P3 and P4) and native site (P5 and P6) to confirm the integration of *psbA2* at each of the two loci on the chromosome.

Primer	Sequence (5′ → 3′)	Location
Neutral site (slr0168) upstream (P3)	GGACCATTCTCTGGATCATTGCC	Homologous to the upstream flanking region of the neutral site
Neutral site (slr0168) downstream (P4)	CAGATTGCCTTTGACAACAATGTGG	Homologous to the downstream region of the neutral site
Native *psbA2* (slr1311) upstream (P5)	GCGTTCCAGTGGATATTTGCTG	Homologous to the upstream region in the native site of *psbA2*. Binds 73 bp upstream
Native *psbA2* (slr1311) downstream (P6)	CA GAT GTC GTT GCT GGT TACA	Homologous to downstream native site. Binds 537 bp downstream

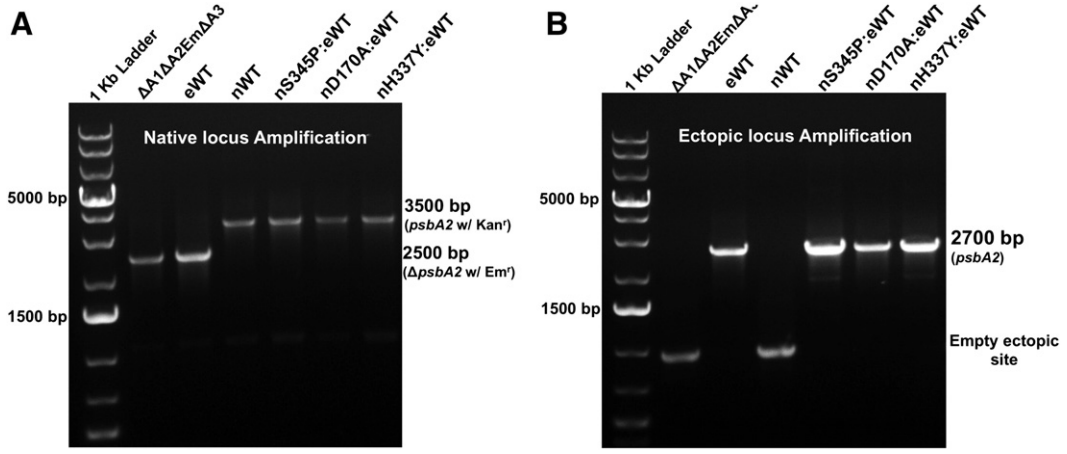

Fig. 2. Dual integration of *psbA2* in dual D1 strains. (A) Native site amplification of strains wherein lanes 2 and 3 (2500 bp) serve as negative controls with the absence of *psbA2* (Em^R), and lane 4 (3500 bp) is positive control with *psbA2* and upstream Kan^R. Lanes 5 through 7 (3500 bp) show integration of *psbA2* at native site. (B) Shows integration of *psbA2* at the ectopic locus. Lanes 2 and 4 (1000 bp) are negative controls with empty neutral site; lane 3 (2700 bp) serves as positive control and lanes 5 through 7 (2700 bp) show *psbA2* at the ectopic locus.

The total variable fluorescence was evaluated with the equation $F_v = (F_t - F_0) / F_0$, where F_t is the fluorescence at time t and F_0 is the lowest level of fluorescence yield obtained as the average yield of a sequence of four weak measuring flashes applied before the first saturation flash. The maximal values of variable fluorescence obtained were normalized by using the formula $F = [(F_v - F_0) / (F_m - F_0)]$ for an appropriate comparison between the control and mutant strains.

2.4. Isolation of thylakoid membrane complexes

A micro scale thylakoid membrane isolation procedure (modified from Komenda et al., 2002 [5]) was used for biochemical analyses. Cultures were grown to mid-exponential phase in 100 ml volumes with continuous shaking under 40 μmol m^{-2} s^{-1} of flux intensity. Cells with at least 250 μg total chlorophyll were harvested and resuspended in 25 mM Tris–HCl (pH7.5) to adjust the chlorophyll concentration to 1 mg/ml. Protease inhibitors were added to a finally concentration of 1 mM and incubated in the dark for 10 min. Cells were broken using Zirconium beads with a diameter of 0.1 mm in 1.5 ml microfuge tubes using a Mini BeadBeater (Biospec Products, Bartlesville, OK, USA). After a low speed centrifugation the cell supernatant was collected and centrifuged at 20,000 ×g for 30 min at 4 °C to obtain isolated thylakoid membranes. Thylakoid membranes were resuspended in 25 mM MES–NaOH (pH6.5) with 25% (v/v) glycerol, 10 mM $CaCl_2$ and 10 mM $MgCl_2$ and used for further biochemical analyses.

2.5. SDS–polyacrylamide gel electrophoresis and Immunoblot analyses

PSII proteins were analyzed by resolving thylakoid membranes using denaturing gel polyacrylamide electrophoresis. Proteins were typically resolved on a linear 12%–20% denaturing gradient polyacrylamide gel containing 6 M Urea [24]. The procedure for sample preparation was modified from Komenda et al., 2002 [5]. 5 μg of total Chl was used and samples were solubilized in 25 mM Tris–HCl (pH 7.5) with 2% sodium dodecyl sulfate (SDS), 2% dithiothreitol (DTT) and 12.5% glycerol. These were incubated at room temperature for 60 min for maximum solubilization and loaded on an equal chlorophyll basis.

To probe the accumulation of D1 protein in the thylakoid membrane samples, proteins resolved by PAGE were transferred onto a PVDF membrane (BioRad) using a semidry blotting apparatus (BioRad semidry western transfer assembly). The blots were probed using rabbit anti-PsbA (D1) antiserum (Agrisera, Sweden). The blot was prepared for development using SuperSignal West Pico chemiluminescence kit (Thermo Scientific, Rockford, USA).

2.6. In vivo pulse radiolabeling using ^{35}S (L-Cysteine)

The protocol devised was adapted from Nickelsen et al., 2004 [25] and Komenda et al., 2005 [26]. Cultures from mid-exponential phase were harvested and resuspended in BG-11 [without sulfur] medium. The chlorophyll concentration was adjusted to 100 μg/ml and a total of 2 ml culture was aliquoted onto a six well plate. The cells were kept in constant shaking under 200 μmol m^{-1} s^{-1} for 20 min. Cells were pulsed for 20 min with 300 μCi of ^{35}S[Cys] during this period the light intensity was immediately increased to 400 μmol m^{-1} s^{-1}. Cold cysteine was added at a concentration of 2 mM and the light intensity was reduced back to 200 μmol m^{-1} s^{-1} for 2 min. Cells from the 6-well plate were transferred to a microfuge tube and were processed for micro scale thylakoid membrane isolation as described in Subsection 2.4.

Labeled thylakoid membranes were loaded on an equal chlorophyll basis and revolved on a 6 M Urea 12%–20% SDS denaturing PAGE. Proteins resolved by PAGE were transferred on a PVDF membrane (BioRad) as described in Subsection 2.5. Dried blot was used for exposure of an autoradiography film (Kodak Biomax) for approximately 12 h prior to development of film.

2.7. Photoinhibition and recovery

Experimental cultures were diluted and adjusted to an $O.D._{750nm}$ of 0.7 and returned to grow at an intensity of 40 μmol m^{-2} s^{-1} for 1 h before the high light treatment to induce photoinhibition. The chlorophyll concentration of the cultures was determined to be ~6 μg/ml. Photoinhibition experiments were conducted in small scale using test tubes (~75 ml, 2.5 cm diameter). These culture tubes were immersed in a circulating water bath maintaining the temperature at 30 °C with constant bubbling of air enriched with 3% CO_2 throughout the experiment. Cultures were exposed to 1100 μmol m^{-2} s^{-1} incident halogen light throughout the high light treatment. Lincomycin (100 μg/ml) was added to stop protein synthesis in order to observe net photodamage during photoinhibition. Sample was withdrawn every 15 min over a period of 60 min high light treatment before returning the experimental cultures to a lower light intensity of 40 μmol m^{-2} s^{-1} to monitor the recovery. For repair characteristics, photoinhibition was performed without lincomycin and recovery was observed for 90 min by determining variable fluorescence at 15-minute intervals.

3. Results

3.1. Parallel expression of two copies of psbA2

The genetic system was designed to express two alternative *psbA2* genes with different rates of D1 turnover in parallel for the accumulation of heterogeneous population of PSII in the cell. The observed phenotype with respect to PSII activity in the dual D1 strains would lead to insights into the existence (if any) of selectivity towards damage prone subunits during D1 degradation. Fig. 1 depicts the overall rationale adopted for this study that allowed us to address the alternate hypotheses targeted versus generalized repair using a single genetic system. The possible phenotypes for the different hypotheses as illustrated in Fig. 1 are predicted to either accumulate a higher percentage of active RCs contributing to a high PSII activity or accumulate both active and inactive RCs resulting in low PSII activity. A targeted repair towards aberrant complexes would selectively replace these mutant *psbA2* preventing the accumulation. Thereby, resulting in a higher population of PSII with WT *psbA2* corresponding to high PSII activity. On the other hand, a generalized repair would trigger an overall increase in the rate of D1 turnover. This would lead to incorporation of both mutant and WT into PSII complexes. Thus, having higher proportion of non-functional PSII centers in the cell leading to a lower PSII activity.

The construction of the genetic system was based on the expression of well-characterized D1 point mutants that have assembly defects or a higher D1 turnover or have increased sensitivity towards high light [11,17–20] in parallel with WT D1 (eWT) (Table 1). The eWT strain expresses a WT from an ectopic site on the *Synechocystis* chromosome. This strain was described previously to have a native-like expression [12]. The construction of the dual D1 strains has been described in Subsection 2.2. The integration of mutant *psbA2* at the native site and the WT *psbA2* at the ectopic site in all the dual strains was performed by amplification of the entire native and ectopic locus in all strains (Fig. 2). For the native locus amplification (Fig. 2A), a 3500 bp DNA fragment corresponding to the *psbA2* gene plus downstream Kan^R cassette was observed in the last four lanes. The strains ΔA1ΔA2EmΔA3 and eWT (lanes 1 and 2) were used as negative control and each represent the deleted native locus wherein the *psbA2* gene has been replaced with erythromycin resistance cassette. The nWT (lane 3) was used a positive control for the presence of the *psbA2* with Kan^R cassette and was produced by re-introducing the wild-type *psbA2* allele into the recipient ΔA1ΔA2EmΔA3 strain thereby restoring PSII function. The ectopic locus amplification in Fig. 2B shows the full-length *psbA2* gene (2,700 bp) in the last three lanes corresponding to three dual expression strains. The full length 2700 bp corresponds to 500 bp upstream and downstream flanking region, 400 bp of native *psbA2* promoter, 1083 bp WT *psbA2* coding region and 200 bp native terminator [12]. The empty ectopic locus of 1000 bp was observed in ΔA1ΔA2EmΔA3 and nWT strains (lanes 1 and 3). The amplified products from native and ectopic loci of each dual D1 strain were sequenced to verify the introduction of point mutation at the native location and WT sequence at the ectopic location.

3.2. Estimation of relative PSII activity in single and dual D1 strains

PSII activity relative to eWT was estimated in all the strains based on variable fluorescence measured using a dual modulation kinetic fluorometer as described in Subsection 2.3. This assay provides an estimate of the relative concentration of PSII complexes that are assembled and capable of light-induced charge separation. Specifically, the changes in the chlorophyll fluorescence induced by a single saturating flash in the presence of DCMU provides an estimate of the percentage of PSII centers that can undergo charge separation to form a $P680^+Q_A^-$ state. The $P680^+Q_A^-$ represents a high fluorescence state and the rate of decay of the high fluorescence state reflects the assembly status of the donor site (Mn_4CaO_5). A functional Mn_4CaO_5 cluster can be oxidized by primary donor $P680^+$ via Y_z thus, the high fluorescence state is relatively more stable and the lifetime of the high fluorescence state is, approximately 1 s [19,27]. In the absence of a fully assembled functional Mn_4CaO_5 cluster, the decay of the high fluorescence state is expected to be much faster resulting from the charge recombination between Q_A^- and oxidant localized in the $P680^+/Y_z$ ensemble. This charge recombination and concomitant decay of the high fluorescence state occurs in about 10 ms [19,27]. This is due to the inability of the Mn_4CaO_5 in providing an electron to the $P680/Y_z$, creating an electron hole in $P680/Y_z$ causing the Q_A^- to rapidly recombine with $P680/Y_z$. Comparison of mutants with wild type variable fluorescence yields the percentage of functional PSII centers for each strain. Oxygen evolution rates for mutants were also determined using a Clarke electrode and it correlated with the variable fluorescence data (data not shown). Table 3 lists the strains with their variable fluorescence and percentage of active PSII centers relative to eWT.

3.3. Fluorescence kinetics in D1-nS345P and nS345P:eWT

D1-S345P re-created in the markerless triple *psbA* deletion background was observed to have a light-sensitive phenotype and could be cultured only under photoheterotrophic conditions in the presence of 5 mM glucose. This observation was consistent with previous characterizations reported in the literature [11,28]. The mutation D1-S345P disrupts the cleavage site for the carboxy terminal protease A (CtpA) and it is thus unable to cleave the carboxy terminal extension required for the assembly of Mn_4CaO_5 [17]. Therefore, the strain is expected to synthesize only the iD1 form bearing the C-terminal extension of 8 amino acids [11,29].

The relative PSII activity in *S345P* estimated from maximal variable fluorescence was 5% of WT (Table 3). PSII activity reported from previous studies shows a variation from 5% to 25% [11,20,28]. Minor differences in the PSII activity could be attributed to growth conditions or the new background strain (marker-less triple *psbA* deletion strain, ΔA1ΔA2EmΔA3). The fluorescence kinetics in D1-S345P was very different from eWT and *nS345P:eWT* (Fig. 3A & B red). The amplitude of variable fluorescence for S345P is very low with only a small fraction of PSII centers capable of charge separation. Due to lower amplitudes, the decay components could not be analyzed for this study. But from previous characterization elsewhere it is known that D1-S345P incorrectly assembles the Mn_4CaO_5 cluster [17,28]. The charge separated state $P_{680}^+Q_A^-$ corresponding to high fluorescence is much lower in the mutant even in the presence of reduced Q_A^-; this is due to a rapid quenching from P_{680}^+. An increased chlorophyll fluorescence quenching is indicative of incomplete reduction by the Mn_4CaO_5 due to a disruption of the donor side of PSII. The mutant strain has been shown to contain photooxidizable Mn that causes the reduction of Y_Z^+ [28].

Table 3
Characterization of PSII activity in all the single and dual D1 expression strains. Columns represent *Fv* (variable fluorescence) and relative PSII activity. F_v was evaluated based on the maximal variable chlorophyll *a* fluorescence (calculated as $(F_{max} - F_0) / F_0$) for each strain measured in the presence of 10 μM DCMU where F_0 is the basal fluorescence yield for a sample before exposure to actinic light and F_{max} is the maximal fluorescence yield that each sample reaches after the actinic light is turned on. The relative PSII activity as F_v for each strain was calculated as percent (%) WT. Values represented are an average of five independent experiments.

Strains	F_v	PSII activity (% WT)
nWT	0.81 ± 0.07	100
eWT	0.81 ± 0.07	100
D1-nS345P	0.04 ± 0.01	5
D1-nD170A	0.21	26
D1-nH337Y	0.04 ± 0.01	5
nS345P:eWT	0.78 ± 0.05	97
nD170A:eWT	0.67 ± 0.08	83
nH337Y:eWT	0.71 ± 0.11	89

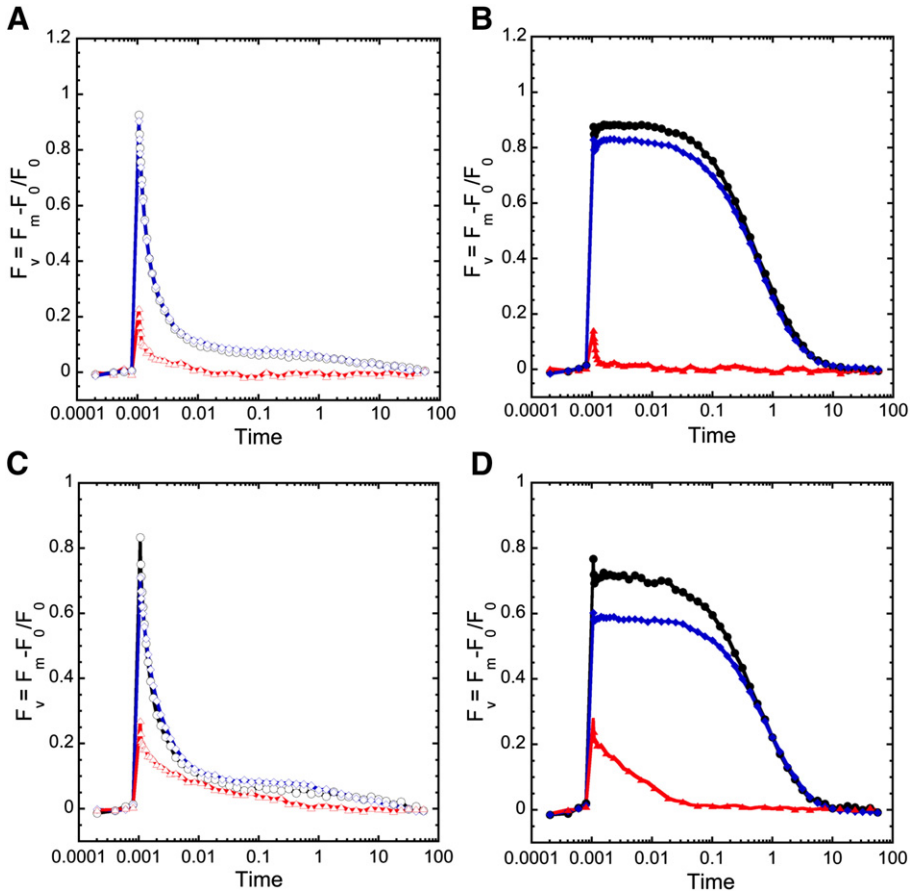

Fig. 3. Q_A reoxidation kinetics of dual expression strains in comparison with WT and single D1 mutants. *Top panel* shows decay kinetics in eWT (black), nS345P (red) and nS345P:eWT (blue) in the absence (A, open symbols) and presence (B, closed symbols) of 10 μM DCMU. *Bottom panel* represents the decay kinetics observed in nD170A (red) and nD170A:eWT (blue) in the absence (C, open symbols) and presence (D, closed symbols) of 10 μM DCMU. Values plotted are calculated as $(F_m - F_0)/F_0$ wherein F_0 corresponds to the average fluorescence yield of first four weak measuring flashes given to each sample before exposure to actinic flash.

In the dual D1 strain *nS345P:eWT*, when wild type was expressed in parallel, a dramatic increase in the fraction of PSII centers capable of successful charge separation was observed. The amplitudes of variable fluorescence are comparable to eWT and accumulate 97% of PSII centers (Table 3). The fluorescence decay kinetics both in the presence and absence of DCMU suggests the presence of a fully functional Mn_4CaO_5 and accumulates predominantly WT PSII centers (Fig. 3A & B blue). With no apparent kinetic differences, it can be implied that a fraction of PSII complexes with S345P-D1 form are either negligible or absent.

3.4. Fluorescence kinetics in D1-nD170A and nD170A:eWT

D1-D170A is a mutation at the donor side of PSII that affects the early stages in the assembly of Mn_4CaO_5 [19,27]. The mutant has been shown to accumulate 70% of PSII centers. In our characterization, the PSII activity was observed to be 25% of WT (Table 3). This mutant is photoheterotrophic and was maintained in the presence of 5 mM glucose in 10–15 μmol m^{-2} s^{-1} of flux intensity.

The kinetics of Q_A reoxidation was measured in *D170A* both in the presence and absence of DCMU (Fig. 3C & D red). A faster decay in fluorescence was observed in the presence of DCMU due to the lack of photooxidizable Mn ions for the reduction of Y_z^+ resulting in a faster charge recombination between Q_A^- and Y_z^+. Earlier observations also show an increased quenching of fluorescence due to a slow reduction of Y_z^+ from an alternate electron donor (P_{680}^+). The decay observed in Fig. 3C could be attributed to this quenching of fluorescence.

The amplitudes of total variable fluorescence were much higher when WT *psbA2* was expressed in parallel with D170A *psbA2* in *nD170A:eWT*. A 70% increase in the proportion of PSII centers capable of undergoing charge separation was observed compared to D170A (Table 3). The decay of fluorescence in the presence and absence of DCMU essentially resembles the *eWT* suggesting an accumulation of WT D1 PSII centers in the dual strain *nD170A:eWT* (Fig. 3C & D blue). Interestingly, kinetic analysis (not shown) indicates that virtually none of the accumulated PSII contains the aberrant charge recombination component in the dual strain *nD170A:eWT* suggesting that the faster turning over mutant D1 protein no longer accumulates when functional D1 protein is simultaneously available.

3.5. Expression of iD1 and D1

As noted,the S345P mutant cannot process the C-terminal extension of the nascent D1 protein, consequently the 8 amino acid C-terminal extension remains intact in the mutant as iD1. Partially assembled complexes with iD1 have been shown to have a higher D1 turnover rate (~15 min) [11]. The iD1 forms being longer than the mature D1 form run slower and can be resolved using SDS–Urea polyacrylamide gel electrophoresis.

The parallel expression of S345P and WT D1 in the *nS345P:eWT* strain was evaluated for the co-expression of iD1 and D1. Thylakoid membranes isolated from *nS345P:eWT* were compared with eWT and D1-S345P on a 12–20% SDS 6 M urea denaturing polyacrylamide gel. Samples were loaded on an equal chlorophyll (Chl) basis of 0.5 μg Chl and the D1 and iD1 forms were probed with antisera against PsbA. Fig. 4 shows the D1 and iD1 accumulation in the thylakoid membranes of these strains. The observation of less accumulation of iD1 form in the mutant D1-S345P is consistent with lower PSII content as observed in variable fluorescence measurements (Table 3). This is also in

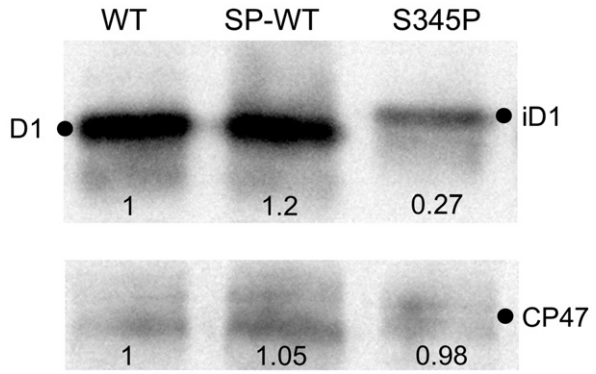

Fig. 4. Accumulation of D1 and iD1 by immunoblot analysis against PsbA (D1). Thylakoid membranes isolated from eWT, *nS345P:eWT* (SP-WT) and nS345P were resolved on a 12%–20% SDS 6 M Urea denaturing PAGE and probed with antibody against PsbA (D1). Lanes were loaded on an equal chlorophyll basis with 5 μg Chl. Bottom panel shows the detection of CP47 that was used as a loading control. The blot obtained was analyzed using Image J analysis software and the relative density for each band listed on the blot was calculated using eWT as a reference.

accordance with previous biochemical analyses of D1-S345P [11]. The accumulation of D1 in the dual strain *nS345P:eWT* was very similar to that of eWT. However, iD1 could not be detected in *nS345P:eWT*. This would suggest that PSII complexes in *nS345P:eWT* are primarily consisting of WT-D1 protein and there are only fewer PSII complexes with D1-S345P (iD1) incorporated, which is beyond the limit of detection by immunoblotting. Therefore, it could be concluded that S345P-D1 is either not synthesized or they are degraded rapidly due to higher rate of D1 turnover and do not accumulate.

3.6. In vivo pulse labeling using ^{35}S[Cysteine]

To confirm the synthesis of iD1 and D1 in nS345P:eWT we observed the incorporation of ^{35}S[Cys] by in vivo pulse labeling. Isolated thylakoid membranes from cells labeled with ^{35}S[Cys] were resolved on PAGE. Fig. 5 compares the levels of ^{35}S[Cys] incorporated in iD1 and D1 after a 20 minute pulse in nS345P:eWT along with eWT and D1-S345P. PsbA triple deletion strain (ΔPsbA) was used as negative control. An equal incorporation of ^{35}S[Cys] in iD1 and D1 indicates the synthesis of both WT and S345P forms of the D1 subunit in the dual D1 strain. Hence, S345P-D1 protein is being synthesized but does not maintain steady state levels due to higher rate of D1 turnover. The identity of labeled band shown in ΔPsbA strain is not clear and, although in the membrane fraction, could not be D1 since the *psbA* genes are entirely deleted.

3.7. Effect of FtsH2 deletion on accumulation of iD1

FtsH a member of the ATP dependent AAA family of proteases has four homologues in *Synechocystis*. FtsH2 (Slr0228) has been shown to be involved in early stages of PSII repair by proteolytically removing D1 subunits [30]. It has been observed that FtsH recognizes the N-terminus of D1 protein to initiate the D1 degradation process [7]. When FtsH2 was knocked out from nS345P:eWT, the PSII activity was decreased by 20% (data not shown). This was expected because with the inability to undergo PSII repair these strains were accumulating damage.

To examine the effect of FtsH deletion on the expression of D1 and iD1, thylakoid membrane samples from *eWT*, nS345P, *nS345P:eWT* (SP-WT) and ΔFtsH-SP-WT were resolved on a 12%–20% SDS 6 M Urea denaturing gel (Fig. 6).

The accumulation of D1 and iD1 was observed using antisera against D1. The relative intensity of the D1 protein band indicates similar levels of accumulation across different samples whereas iD1 was accumulated to only ~25% of mature D1 levels. This was consistent with previous observation in Fig. 4. An accumulation of iD1 and D1 was observed in ΔFtsH-SP-WT indicating co-expression of both mutant (S345P) and wild type (WT) D1 proteins in the dual D1 strain. Additionally, absence of the iD1 band in the nS345P:eWT strain suggests that the S345P mutant form of D1 is being synthesized but it has a higher rate of turnover therefore does not accumulate to higher levels for visualization on an immunoblot.

Taken together, the findings from the parallel expression experiments described above are consistent with the existence of a mechanism that selectively degrades aberrant forms of the D1 proteins, either because the proteins is prone to damage (D1-H337) or because is structurally altered due to failure to be processed (D1-S345P) or lacking key Mn-binding residues (D1-D170A).

3.8. Role of conserved cysteine residues in D1

Previous work has established that the N-terminus of D1 protein is essential for its degradation and truncation of the N-terminus leads to a light-sensitive phenotype due to the failure to replace the damaged protein [7]. The N-terminus contains a highly conserved cysteine at position 18 (D1-C18), which could have a functional role in the thiol regulation of the repair process which has been previously shown to be affected by thiol modifying agents [31]. To begin to examine this possibility, the D1-C18A and D1-Cysless mutation (four native Cys18, Cys47, Cys125 and Cys144 all replaced by Ala) was constructed and its effect upon the damage and repair process was examined by exposing mutant and wild-type to photoinhibitory high light for 60 min followed by a return to lower growth light intensities to allow for recovery of PSII activity (Fig. 7).

The Q_A reoxidation kinetics of D1-C18A and D1-Cysless was similar to nWT indicating the functional PSII complexes capable of successful

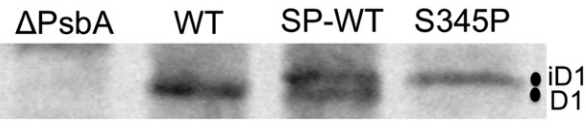

Fig. 5. Synthesis of iD1 and D1 analyzed by in vivo pulse using ^{35}S[Cys]. Thylakoid membranes isolated from ΔPsbA, eWT, nS345P:eWT (SP-WT) and nS345P pulse labeled with ^{35}S[Cys] were resolved on 12–20% 6 M denaturing PAGE and transferred onto PVDF membranes. Samples were loaded on an equal chlorophyll basis with 0.5 μg chl. Dried blots were used for exposure onto autoradiography film for 14 h prior to development.

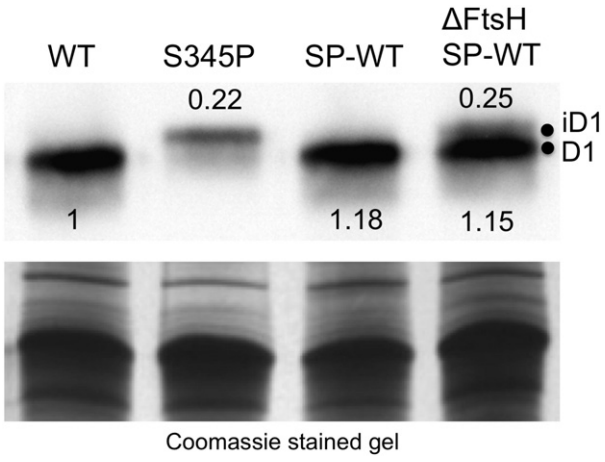

Fig. 6. Effect of FtsH protease deletion on the accumulation of D1 and iD1. Thylakoid membranes from WT (*eWT*), *S345P*, SP-WT (*nS345P:eWT*) and ΔFtsH-SP-WT were resolved on a 12%–20% SDS denaturing PAGE and probed with antibody to D1. Lanes were loaded on an equal chlorophyll basis with 5 μg Chl. Bottom panel shows the corresponding Coomassie stained gel as a loading control. Bands were analyzed using Image J analysis software and the relative density for each as listed on the blot was calculated using WT as a reference.

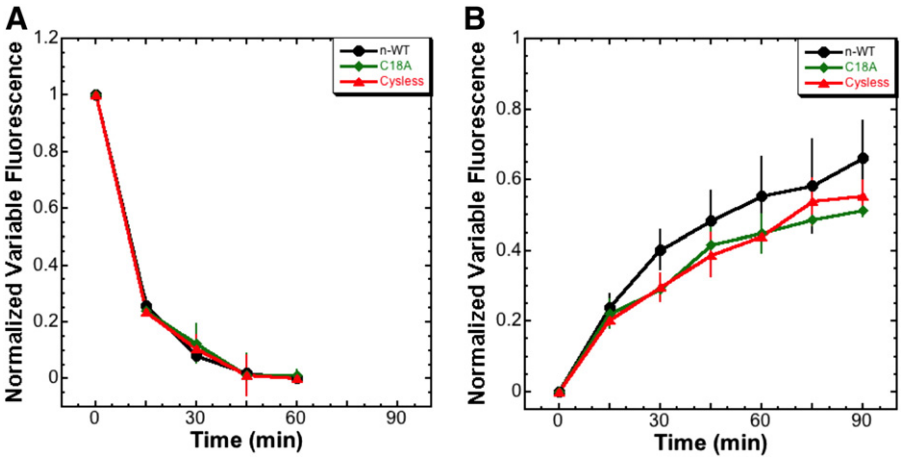

Fig. 7. Photoinhibition (A) and repair characteristics (B) of D1-C18A (green) and D1-Cysless (red) when compared to nWT (black). (A) Decrease in variable fluorescence with time recorded when nWT (black), D1-C18A (green) and D1-Cysless (red) were subjected to high light intensity (1100 μmol m^{-2} s^{-1}) for 60 min. Lincomycin (100 μg/ml) was added to stop any repair during photoinhibition. (B) An increase in variable fluorescence as a function of time observed once the cells, without lincomycin addition, are returned to lower light intensity (40 μmol m^{-2} s^{-1}). The variable fluorescence values were normalized for comparison. Graphs represent average of three independent experiments and error bars indicate corresponding standard errors (n = 3).

charge separation (data not shown). Therefore, replacement of cysteines does not alter the donor side of PSII and results in the formation of PSII complexes that are functional. Photoinhibition characteristics in the presence of lincomycin allowed us to analyze the rate of photodamage in the Cysless mutants and WT. Fig. 7A suggests that the rates of photodamage are identical to that of nWT, whereas recovery of PSII activity (in the absence of lincomycin) was hampered in D1-C18A (green) and D1-Cysless (red) (Fig. 7B). The rates of photodamage and recovery shown here do not take into account any possible non-photochemical fluorescence quenching differences. However, Cysless strains appeared more inhibited in damage and repair compared to WT even under normal light (40 μmol m^{-1} s^{-1}) in the presence and absence of lincomycin (data not shown). Slow recovery in Cysless mutants (Fig. 7B) implies inefficient D1 replacement during PSII repair. The rates of recovery for C18A and Cysless were similar indicating that replacement of other cysteines in D1 does not have any additional effect on the PSII repair process. Therefore, C18A replacement could be a plausible reason for the slower recovery observed in the D1-Cysless strain. Since, Cys 18 is highly conserved in the majority of oxygenic photosynthetic organisms, this residue might play a role in displaying the damaged D1 signal. Thereby providing a plausible explanation for the inefficient repair characteristics observed in D1-C18A. However, further investigation will be required to confirm this finding.

4. Discussion

The parallel *psbA* expression system builds on the ectopic WT strain constructed previously [12]. This genetic system enables the co-expression of alternative forms of D1 protein from identical promoters, thereby allowing tests on the possible alternative cellular fates of the co-expressed *psbA* alleles. The overall purpose of this dual D1 expression system was to address the underlying question whether PSII complexes damaged during photoinhibition and/or prone to high turnover are specifically targeted for repair? Taking into account that an alternate hypothesis exists wherein photoinhibition elicits a general up-regulation in the rate of D1 replacement process such that damaged and undamaged subunits both have increased turnover probabilities. In principle, either alternative could account for higher rates of D1 turnover under photoinhibitory conditions [3–9], although a targeted repair mechanism would seem to be the metabolically more efficient strategy.

The strategy for this parallel expression approach was to co-express damage prone mutants with faster rates of D1 turnover and a wild type in order to accumulate heterogeneous populations of PSII (Fig. 1). By introducing heterogeneity in a single strain, we can monitor if the cell specifically targets mutant PSII complexes for repair. The genetic system developed enabled us to address the alternate hypotheses of targeted versus generalized repair by evaluation of the PSII activity in these parallel expression strains.

For the successful construction of dual D1 strains, turnover-prone D1 mutants were cloned into the native locus and WT was cloned into the ectopic locus of the chromosome. By using this approach we ensured the native expression of mutant *psbA2*, as it was being cloned into the original site in the chromosome. Cloning the wild type *psbA2* instead of a mutant into the ectopic site prevented any untoward alteration in the phenotype of the mutant that would otherwise be attributed to differences in the gene location. Both the genes (mutant and WT) were under the control of native *psbA2* promoter and shared the same 5′ and 3′ UTR (untranslated region). This increased the likelihood that both genes are being regulated similarly and any difference in the expression of one gene would be carried over to the other gene as well. Thus, both genes were constructed with an intention to maintain similar gene regulation including transcription and translation.

Turnover-prone D1 mutants for dual D1 constructions were chosen primarily based on their sensitivity to photoinhibition (D1-H337Y), incorrect assembly of Mn_4CaO_5 (D1-D170A) and increased rate of D1 turnover (D1-S345P). D1-S345P, C-terminal extension mutant that does not undergo D1 C-terminal processing and has a 8 amino acid extension causing the Mn_4CaO_5 to not assemble correctly thereby has an increased D1 turnover rate [17]. This mutant has been shown previously to have lower accumulation of PSII centers compared to wild type [17,28]. Similarly, D1-D170A also affects the donor side of PSII. This mutant cannot assemble the Mn_4CaO_5 cluster and has been shown to have a D1 turnover halftime of ~15 min [4,19,28]. Additionally, D1-H337Tyr is also a mutation causing the abberrant assembly of Mn_4CaO_5. This mutant has been suspected to create reactive oxygen species making this mutant extremely light sensitive [20].

4.1. Towards targeted repair mechanism

When these single D1 mutants were expressed from the native site alone their PSII activity measured as variable fluorescence was very low, indicative of decreased accumulation of the charge-separating PSII RCs (Table 3). The values of relative PSII activity observed were lower than those previously reported [18,20,22]. This could be due to differences in light and growth conditions, although differences in strain background cannot be excluded. When a WT D1 was expressed in

parallel, all the three dual D1 *strains nS345P:eWT*, *nD170A:eWT* and *nH337Y:eWT* restored the phenotype of nearly WT PSII activity (Fig. 3 and Table 3). Relative PSII activity determined based on the total variable chlorophyll *a* fluorescence suggests an increased proportion of PSII complexes with WT D1 protein incorporated. The kinetics of decay of the high fluorescence state in the presence and absence of DCMU also indicates a large population of PSII centers undergoing successful charge recombination between the Q_A^- and Mn_4CaO_5 in the S_2 state (in the presence of DCMU, Fig. 3B and D) and a large proportion of PSII complexes undergo Q_A^- to Q_B transfer (in the absence of DCMU, Fig. 3A and C). The kinetics of Q_A reoxidation was similar to a large extent in all the dual D1 strains suggesting a predominant accumulation of PSII RCs with WT D1 protein. This would imply that either there is no accumulation or very less accumulation of mutant PSII RCs. Accordingly, we ask why are the mutant PSII complexes not being accumulated?

As reasoned earlier (Fig. 1), a high PSII activity in dual D1 strains is most consistent with a targeted repair mechanism of the damage prone PSII complexes. This can explain the absence or lower amounts of mutant PSII complexes. Another less plausible alternative could be that introduction of two copies of a gene has some regulatory effect on the mutant *psbA2* causing the gene to be silenced. This seems highly unlikely as the mutant *psbA2* is being expressed from the native locus and we would be expecting an effect (if any) on the WT expressing from the non-native (ectopic) location. Constructs were designed with identical gene regulatory regions to enable similar effects on transcription and translation on both loci. Nevertheless, any effect due to transcript levels cannot be excluded. Nevertheless, major changes in transcript levels are not expected, as D1 synthesis rates do not show differences among strains (Fig. 5).

A mutant that accumulates an increased proportion of non-functional RCs would be more promising to address this hypothesis. D1-D170A was able to accumulate 25% of WT PSII under the conditions used for this study. The dual strain nD170A:eWT also accumulated 85% of PSII RCs relative to WT. When the decay kinetics of fluorescence was analyzed by normalizing the amplitudes, a small fraction of PSII RCs was observed to undergo a slightly faster decay (data not shown). This faster decay component was consistently observed and can be attributed to the faster decay in the mutant D1-D170A. Yet this kinetic component was absent when WT D1 is expressed in parallel suggesting that the WT D1 protein is more stably associated with a finite pool of PSII complexes or that mutant D1 is rapidly removed and preferentially replaced with the WT D1.

Analysis of D1 and iD1 accumulation in the thylakoid membranes isolated from *nS345P:eWT* did not show an accumulation of the iD1 form (Fig. 4). The amount of iD1 accumulated in S345P was approximately 30% of that of the D1 protein observed in WT. A lower accumulation of iD1 has been reported previously [11]. However, there was no iD1 observed in the dual again. Again, suggesting that PSII complexes are predominantly associated with WT PSII complexes. This raises that possibility that the observed results that reflect different stabilities of the nascent forms of D1 may be subject to 'quality control' early in the assembly process. However, it also seems likely that the D1-D170A and D1-H337Y mutants are only minimally different from the WT D1 prior to the assembly or attempted assembly of the Mn cluster, which occurs fairly late in the overall assembly process [1,2,32–34]. Therefore, it seems more likely that the observed preferential association of PSII complexes with WT D1 in the dual strains reflects a competition of sites and faster turnover of mutant D1 copies leading to their replacement by the WT D1 form, which is lost more slowly and accumulates at the expense of the mutant form under the conditions used in this study.

In vivo pulse labeling of *nS345P:eWT* showed incorporation of ^{35}S [Cys] in both iD1 and D1 providing an unequivocal evidence for the co-expression of S345P-D1 and WT-D1 in the dual strain. When thylakoid membranes from FtsH2 knockout version of nS345P:eWT were compared with S345P:eWT, an accumulation of the iD1 form was observed in immunoblots resulting from the accumulation of damaged D1 due to the absence of the degradation protease, FtsH2 (Fig. 6). Therefore, by using the FtsH knockout strain it is evident that both genes (mutant and WT) are being co-expressed and the increased PSII levels observed in dual D1 strains (Table 3) and the absence of the iD1 (D1-S345P) form in the dual strain S345P:eWT are due to specific targeting of PSII complexes with D1-S345P incorporated. This mutant form cannot complete the assembly process beyond iD1 and therefore gets turned over rapidly, whereas the PSII complexes with WT incorporated are much more stable and complete the PSII assembly process.

A clue to the possible mechanism of targeting comes from the observation that the mutation of the D1-Cys18 residue results in the impairment of the recovery from photoinhibition, but does not affect the rate of photodamage during the exposure of cells to high light treatment. The result is not dramatic, but points to the possibility that thiol modifications (e.g. glutathiolation or the formation of other disulfide adducts) may be involved in the processing of nascent or damaged D1. Obviously, many questions still exist about the nature of the PSII repair process and additional experiments still need to be performed with the existing dual strain system to fully understand it, yet the results presented here point to the existence of a selective molecular recognition of aberrant PSII targeting those complexes for D1 replacement.

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