ECTOPIC EXPRESSION OF FULL LENGTH D1 PROTEIN AND FACTORS LIMITING PHOTOSYSTEM II (PSII) REPAIR IN *SYNECHOCYSTIS* sp. PCC6803

By

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SYNECHOCYSTIS sp. PCC6803

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DEDICATION

I dedicate my thesis to my beloved Grand parents Mr. and Mrs. Narayana Rao Subbayamma and my aunt Umamaheswari, who wished to see me in this position.

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ABBREVIATIONS AND SYMBOLS

1. PS I…………………………………..………………………..Photosystem I
2. PS II………………………..…………………………………Photosystem II
3. RCC……………………………………………..Reaction center complexes
4. RC…………………………………………………………….Reaction center
5. ROS…………………………………………….Reactive Oxygen species
6. Fv………………………………………………………Variable fluorescence
7. F_{HA-QA}……………………………………...Variable fluorescence with chemicals
8. Fo…………………………………………………………..Initial fluorescence
9. Fm…………………………………………………maximum fluorescence
10. Control strains……………………………………………………….WT, WT*
11. Mutants..MK1, MK2
12. C-terminal...Carboxy terminal
13. N-terminal...Amino terminal
14. OD...Optical density
15. μ…...micro
16. cDNA...Complimentary DNA
17. ml...milli liters
18. mM…………………………………………………………………milli mole
19. ng………………………………………………………….......nano gram

20. °C……………………………………………………………………centigrade

21. Ct……………………………………………………Threshold cycle value

22. PQ...Plastoqinone

23. PQA..Plastoquinone A

24. PQB..Plastoquinone B

25. DCMU....................................... (3-(3,4-dichlorophenyt)- 1,1-dimethylurea)

26. DCBQ...2,6-dichloro-1,4-benzoquinone

27. HA……………………………………………………………..Hydroxyl amine

28. FeCN…………………………………………………………Ferric cyanide

29. OEC………………………………………………Oxygen evolving complex

30. mRNA...messenger RNA

31. K_D...Damage rate constant

32. K_R…………………………………………………….Recovery rate constant

33. µmol ……………………………………………………………micro Einsteins

34. rpm………………………………………………………rotations per minure

35. Xg…………………………………………………………………gravity force

36. mv……………………………………………………………………milli volts

CHAPTER I

Introduction and Literature review

Introduction

Photosynthesis is the primary source of energy on earth. All green plants, algae and some bacteria utilize CO_2, H_2O and light energy from sunlight to convert light energy to chemical energy which can be stored in the form of carbohydrates with the release of molecular oxygen (Govindjee., 1999). These photosynthetic organisms are named as "autotrophic" based on their ability to prepare their own food. The oxygen released by these organisms in the process of photosynthetic electron transport is responsible for the survival of heterotrophic organisms (Gust, D., 1996).

All oxygenic photosynthetic organisms possess two major reaction center complexes (RCC) called Photosystem I (PS I) and Photosystem II (PS II).These RCC are located in chloroplasts in higher plants and algae. As prokaryotes lack sub-cellular organelles, these RCC are present in thylakoid membranes (Govindjee., 1999).

PSII is one of the major multi-subunit complex containing around 30 different subunits and a variety of coenzymes and cofactors. It is embedded in thylakoid membranes in all photosynthetic organisms and utilizes light energy to

split water into oxygen, protons and electrons. The PS II transfers them to plastoquinone (PQ) resulting in the production of molecular oxygen. The PS II complex is responsible for the oxygenic atmosphere on the earth (Whitmarsh et al., 2002). The major proteins D1 (encoded by *psbA*) and D2 (encoded by *psbD*) are present in heterodimeric reaction center core (Bryant.,1994). D1 and D2 proteins co-ordinate redox active components of PS II electron transport chain (Terbst.,1986). High light induces damage to PS II reaction center D1 protein through oxidative damage or inactivation of electron transport (Aro et al., 1993; Walker et al, 1991). This light-induced loss of photosynthetic activity is termed photoinhibition. The rate of photodamage is directly proportional to the light intensity. The damaged D1 protein is replaced by newly synthesized D1 ensuring the survival of the organism (Aro et al., 1993).

When the rate of damage exceeds the rate of repair, damaged PSII complexes accumulates within the thylakoid membranes causing a decline in molecular oxygen evolution (Figgure1.1). Proteases are involved in the removal of damaged D1 protein. The exact mechanism of D1 protein turnover is unknown at this time. The removal of damaged D1 protein takes place with the disassembly of PS II complex and removal of damaged D1 protein followed by insertion of new D1 protein after synthesis (Figure1.1) , resulting in functional PS II (Polle JE et al., 2003).

Damage and Repair cycle of PSII

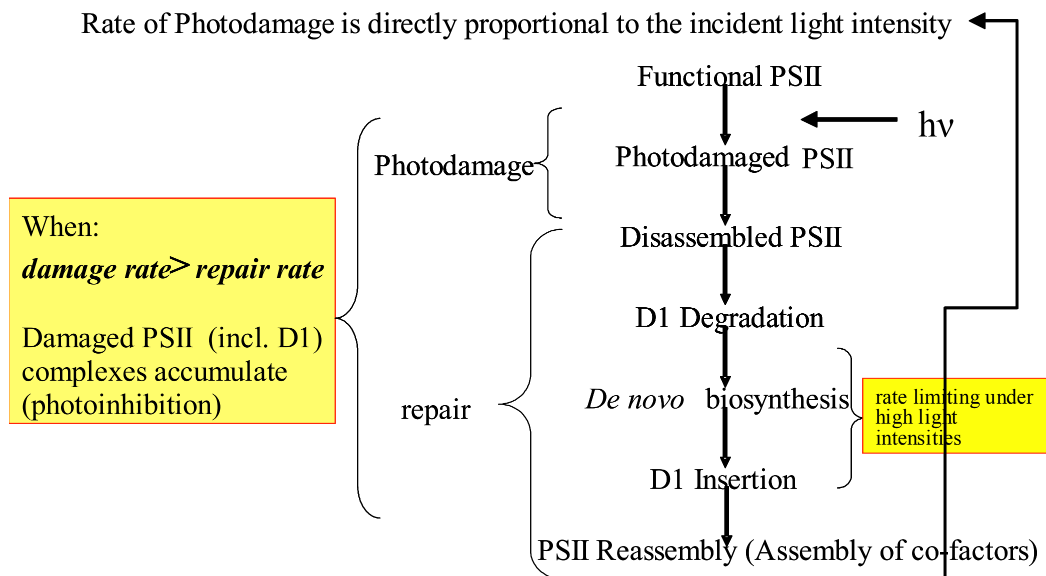


Figure 1.1 Flow chart indicating damage and repair cycle of photosystem II

In my current research i focused on studying the process of turnover of the D1 protein which can be studied in the cyanobacterium *Synechocystis* sp. PCC6803 (*Synechocystis* 6803). This is a model organism for plants as chloroplasts are believed to be evolved by endosymbiosys from cyanobacteria (Kutschera., 2005).

Statement of Biological Problems

- Are the N-terminal cysteine residues of the D1 protein important for the repair of D1 protein in PSII

- Test the hypothesis: the level of transcript limits the rate of repair of D1 protein in PSII.

Literature Review

Photosynthesis

Photosynthesis is the biological process needed for conversion of sunlight into chemical energy and that energy is stored in the form of carbohydrates. This process is responsible for the production of molecular oxygen. The synthesized carbohydrates and oxygen released by these organisms in the process of photosynthetic electron transport is responsible for the existence of most life on earth (Govindjee., 1999 ; Gust, D., 1996).

There are two types of photosynthesis, oxygenic and anoxygenic based on the production of molecular oxygen. In oxygenic photosynthetic organisms like higher plants, algae and cyanobacteria, molecular oxygen is released as a by product of water oxidation. In contrast, anoxygenic organisms like purple and green bacteria, and other photosynthetic bacteria, utilizes inorganic and organic compounds as electron donors without releasing molecular oxygen as a by product (Govindjee., 1999).

PS I and PS II are the two RCC in photosynthetic organisms. These RC complexes are located in chloroplasts in higher plants and algae. As prokaryotes lack chloroplasts, these RC complexes are present in thylakoid membranes (Govindjee., 1999).

In the presence of light, electrons are transferred from Photosystem II to Photosystem I through intermediate carriers. The overall set of light dependent

reactions involves the highly endergonic transfer of electrons from water molecules to $NADP^+$, resulting in NADPH (Govindjee., 1999). In order to drive this endergonic reaction sequence, sun light contains enough energy to be used as a coupler for this pathway. During the electron transfer process, a trans-membrane proton gradient is formed and this is used to drive the synthesis of ATP via ATP synthase. The resultant ATP, together with NADPH, is used to fix CO_2 into carbohydrates through the calvin cycle (Figure1.2).

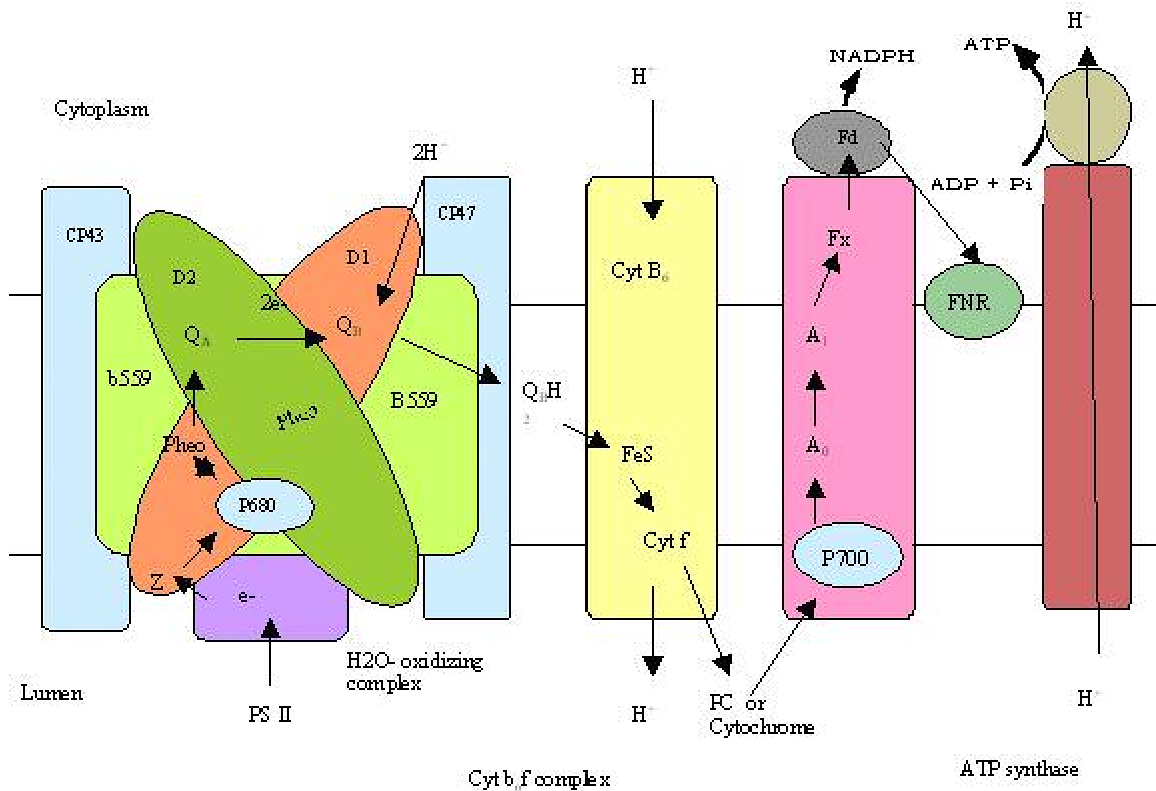


Figure 1.2: Figure representing the energy liberating reactions of Photosynthesis

PS II is the major RC complex of photosynthesis. It contains 30 different subunits, co-factors and coenzymes (Figure1.3). PS II is responsible for the production of the oxygenic environment on earth by splitting water molecules with the help of sun light. It utilizes light energy to split water into oxygen, protons and electrons and transfers them to plastoquinone (PQ) resulting in the production of molecular oxygen, needed for the existence of most life (Whitmarsh and Govindjee., 2002). D1 protein is the key protein of PS II and is located in the heterodimer reaction center core of PS II along with the D2 protein (Bryant., 1994). These D1 and D2 proteins assemble all other components of PS II electron transport chain (Terbst., 1986).

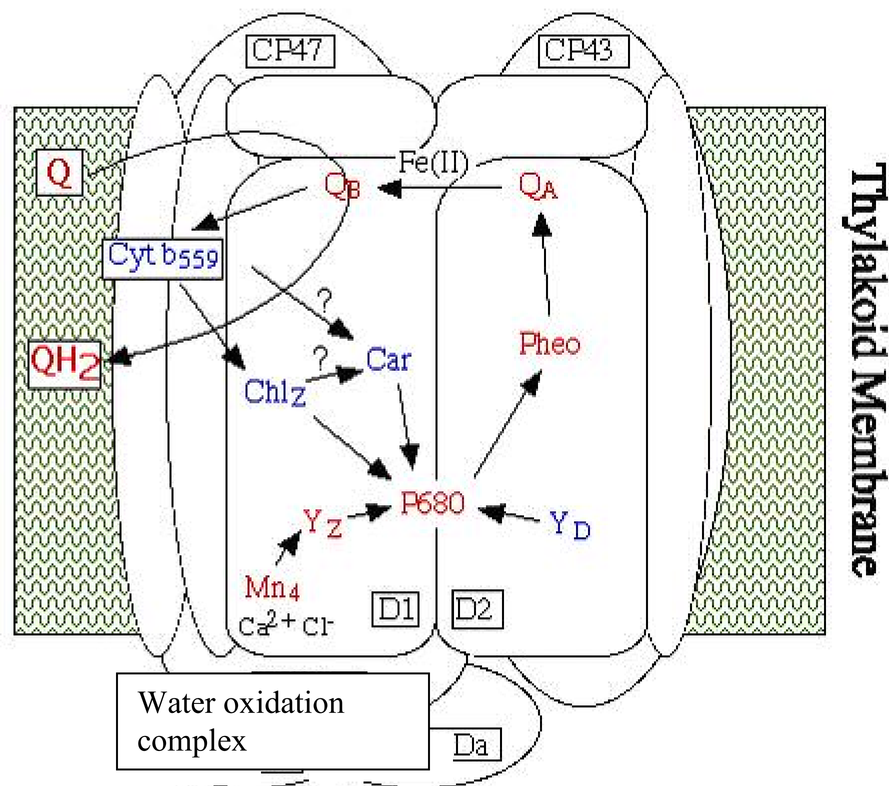


Figure 1.3: Schematic representation of Photosystem II electron transport chain

Source:http://ursula.chem.yale.edu/~brudvig/psii.gif

At the core of PS II, a special pair of chlorophyll molecules known as P680 is located. When P680 absorbs a photon, it becomes $P680^+$ (excited state). An electron transferred from $P680^+$ to pheophytin inturn reduces a plastoquinone molecule called $PQ_{A.}$ PQ_A is a single electron acceptor bound to the D2 protein. The reduced PQ_A transfers this electron to secondary plastoquinone acceptor PQ_B which is a two electron acceptor. After receiving a second electron from PQ_A by the charge separation event at P680, the reduced PQH_2 leaves the site and diffuses through the membrane. From the membrane pool, a new PQ_B molecule occupies the secondary plastoquinone acceptor site. On the donor side the positive hole on $P680^+$ is transferred to a manganese cluster through tyrosine Z. P680+ oxidizes tyrosine Z which inturn oxidizes a cluster of four manganese atoms (Figure 1.3). This metal center is the catalytic site of water oxidation (Bridgette A., 1994 ; Debus R., et al 1986).

In our current research on the PS II D1 protein turnover, we use the model organism *Synechocystis* sp. PCC6803 (*Synechocystis* 6803). It has been used in our field because chloroplasts are believed to have evolved from endosymbiotic photosynthetic bacterium (U. Kutschera., 2005).

Cyanobacteria

Cyanobacteria are oxygenic photosynthetic prokaryotes. The photosynthetic apparatus of these cyanobacteria resembles chloroplasts with the exception of the light harvesting antenna system. These organisms lack chlorophyll b, while depending on chlorophyll a and phycobilisomes for absorbing light energy.

It is believed that the higher plant chloroplast is similar to the modern cyanobacteria having an autonomous genome (SE Douglas.,1991). Cyanobacteria are evolutionarily related to green chloroplasts of higher plants from 16S rRNA, sequencing studies (Stephen et al.,1988). The biochemical and molecular studies provide evidence for the endosymbiotic origin of chloroplasts (SE Douglas.,1994).

Most of the work on PS II and D1 protein has been done on isolated chloroplasts. The thylakoid membrane of cyanobacteria structurally and functionally resembles the photosynthetic membrane of higher plants, studies using either system will reveal much of the same information (SE Douglas.,1991). For our studies we used a unicellular cyanobacterium called *Synechocystis* sp. PCC6803.

Synechocystis PCC6803

The cyanobacterium *Synechocystis* sp. PCC6803 was the first unicellular photosynthetic organism for which a complete genome sequence was solved (Kaneko., 1996). It is extensively studied as a model organism for plants. It grows both photoautotrophycally and photoheterotrophycally and the strain is easily transformable with high transformation efficiency (Matsunaga., 1990). The genomic sequence information was made available in 1996 by Dr. Satoshi Tabata and co-workers at the Kazusa DNA Research Institute. This information is presently available at http://www.kazusa.or.jp/cyano/, called “cyanobase”. The availability of this information helped to elucidate the functional mechanism of

different genes and proteins by gene knockout and mutagenic studies (Y Nakamura et al*)***.**

D1 protein

D1 protein is described as the core protein of PS II given that it associates with the redox active components of PSII electron transport chain (Figure 1.3). It now plays an important functional role in the research of photosynthesis (Terbst.,1986). This protein is highly conserved among all photosynthetic organisms with 80% aminoacid sequence similarity (Jansson et al., 1997). It is a trans thylakoidal protein, containing five transmembrane helices. This protein also has the highest turnover rate, as a photoprotection mechanism against Photodamage from excessive light (Klein et al., 1988).

D1 protein is encoded by the *psbA* genes, in higher plants and algae there is a single *psbA* gene copy where in cyanobacteria, this protein is encoded by multigene *psbA* family (Mohamed et al., 1993). Three *psbA* genes in *synechocystis* sp.PCC 6803 (*psbA1, psbA2, psbA3*) codes for the D1 protein. Out of the three *psbA* genes, *psb1I* shows rare expression in *Synechocystis* sp.PCC 6803 while *psbA2* and *psbA3* together codes for 99% of the total D1 protein. *psbA2* accounts for 90% of D1 protein transcript. (Mohamed et al., 1993).

The information on the synthesis, membrane insertion and the assembly of the D1 protein into PS II complex is available based on the research performed on chloroplasts (Zhang et al., 2000). Given the origin of the

chloroplasts, it is unsurprising the similarities observed in comparison to cyanobacteria.

D1 protein is synthesised as a precursor D1 protein with carboxy terminal extension. The carboxyl-terminal processing protease (CtpA) cleaves the carboxy terminal extension during the maturation of D1 protein (Karnauchov, Herrmann et al., 1997). Processing of C-terminal of D1 protein is important for reassociation of the oxygen evolving complex (Zhang et al, 2000). Translation of the D1 protein takes place on ribosomes bound to thylakoid membranes from mRNA encoded by the *psbA* gene. Replacement of photodamaged D1 protein with a newly synthesized copy maintains the function of PSII under high light (Klein et al., 1988). D1 protein assembles co-translationally into PSII through thylakoid Sec pathway. The D1 depleted PSII acts as a receptor for the newly synthesised D1 protein (Zhang et al., 2000).

Photoinhibition

Photoinhibition is a process of inhibition in photosynthesis due to excessive light. Light is an important factor in photosynthesis to drive the transport of electrons from water to $NADP^+$. This provides the hydrogen's for the reduction of CO_2, by the Calvin cycle. In steady-state photosynthetic conditions, reduction of CO_2 increases linearly with the light intensity. Increases in intensity over a certain threshold results in excess absorption of photons than the capacity of the carbon-fixing reaction which leads to some damaging effects (Kruce., 1998). Above this threshold, the rate of photodamage is directly proportional to light intensity. Photoinhibition impairs the process of photosynthesis by damaging

the proteins and membrane lipids of the photosynthetic apparatus. PS II is the primary site of damage in photoinhibition and is the chief cause of impairment in the electron transport chain. As the D1 of PS II protein is the core component in binding the major PS II electron transport chain cofactors(Figure 1.3), it may not be surprising that illumination of high light damages the D1 resulting in the loss of PS II activity (Mulo et al., 1998). This high light intensity induces damage to the D1 protein through oxidative damage or inactivation of electron transport (Aro et al., 1993), although the precise mechanism has yet to be established.

There are different hypothesis on the mechanism of photoinhibition. The exact mechanism of photosystem II inactivation *in vivo* still needs to be elucidated (Mulo et al., 1998). *In vitro* studies, provided knowledge about two major pathways of photo damage based on acceptor side and donor side photoinhibition.

The mechanism of acceptor side photoinhibition involves damage to the PS II electron transport chain at the level of PQ_A / PQ_B. Exposure to strong light can lead to over reduction of the plastoquinone pool. PQ_B leaves the site resulting in increase of reduced PQ_A half life. The reduced PQ_A fails to conduct electrons and promotes charge separation between $Pheophytin^-$ and $P680^{+}$, leading to the formation of the chlorophyll triplet state. Chlorophyll triplets will react with molecular oxygen forming highly reactive singlet oxygen, which leads to the damage of D1 protein (Vass et al., 1992).

The other type of photoinhibition is donor side photoinhibition, caused due to transient inactivation of electron transfer from water to P680. This process

occurs when the rate of electron donation to $P680^+$, is slower than the removal of electrons at the acceptor side. Oesterhelt, 1995 conducted experiments on the green algae *Chlamydomonas reinhardtii* to detect donor side photoinhibition. This mechanism leads to the formation of $P680^+$ and Yz^+, leading to photoinhibition by causing major damage to the D1 protein of PSII (Bumann et al., 1995).

Oxygen is an important factor for the survival of organisms and at the same time the ROS (reactive oxygen species) which is formed by activation of ground state oxygen can adversely effect cellular components. ROS are generated during exposure of photosynthetic machinery to high light or during limited availability of CO_2 and $NADP^+$. These reactive oxygen species target photosynthetic machinery, where molecular oxygen is produced during photosynthetic electron transport. In acceptor mechanism, reactive oxygen species are indicated to be responsible for photodamage. *In vivo*, these reactive oxygen species were reported to participate in photoinhibition by inhibiting the synthesis of proteins but not directly involved in damage process (Jansen et al., 1993).

Jan M. Anderson, 1998 indicated the light intensity used by researchers for *in vitro* studies is a rare event *in vivo*. The acceptor side photoinhibition where singlet oxygen causes damage to PS II is not entirely relevant *in vivo*. They reported that plants naturally have many photoprotection mechanisms, which prevents them from dangerous symptoms due to light exposure. Waxy coating on leaves and the staking of damaged PS II complexes around D1 protein acts as a self protection from light exposure. In natural sunlight environment, plants are

exposed to single photon, but *in vitro* when thylakoid membranes are used for photoinhibition experiments, natural photoprotection mechanisms are impaired as a result of high photon intensity (Jan M. Anderson., 1998). *In vivo* the oxygen evolved by water splitting is released by a special channel preventing the reaction of singlet oxygen to avoid damage to D1. On the contrary, when isolated thylakoid membranes are exposed to high photon flux, it disturbs the natural environment and the results vary (Jan M. Anderson., 1998). This indicates the mechanisms of photoinhibition are controversial and the exact mechanism, in steady-state to the situations *in vivo* needs to be elucidated.

Recovery

To maintain the activity of photosystem II, the damaged D1 has to be degraded with subsequent replacement of newly synthesized D1. This event restores function of photoinhibited PS II. The PS II activity at any light intensity is a result of balance between the rate of damage and the rate of repair (Figure 1.4). The rate of repair depends on the aggregate rate of degradation of damaged D1, *de novo* synthesis and insertion of newly synthesized D1. At high light intensity the *psbA* mRNA levels increase due to increased transcription rates leading to the initiation of translation resulting in the recovery of damaged PSII. When the light exposed cells transferred from high light intensity to low light intensity, they will restore the function of PS II since repair rates are not exceeded by damage rates. But prolonged exposure of the cells to high light intensity results in irreversible recovery indicating lethal damage to transcriptional and translational machinery (Constant et al., 2000).

Photoinhibited cells can recover only in the presence of low light intensity. The requirement of light for recovery of damaged PS II activity was studied by Constant et al (2000) . They indicated from their experiments photoinhibited cells were unable to recover from damage in darkness even in the presence of glucose. During the initial high light exposure event, the amount of *psbA* mRNA is excessive, indicating higher rates of transcription. In later stages the rate of transcription decreases slowly resulting in photodamage (Constant et al., 2000). Translation inhibitors like lincomycin and chloromphenicol, when used in photoinhibition experiments provides an estimate of damage rate of PSII in the absence of repair. When these protein synthesis inhibitors have been added to the culture during photoinhibition experiments, the cells failed to recover, due to the absence of translation (Komenda et al., 2000) .

Komenda et al (2000), observed D1 and D2 degradation in different strains of *Synechocystis* sp PCC 6803. In their studies, they used six different *Synechocystis* sp.PCC6803 mutant strains. These strains differ with the type of *psbA* gene and location of antibiotic cassette. Pulse-chase labeling with ^{35}S was conducted both in the presence and absence of lincomycin. Results indicated that the level of *psbA* mRNA regulates PS II repair cycle (Komenda et al., 2000). However, other factors have been proposed to limit PSII repair. Damaged PSII center accumulation was observed in thylakoids of plants exposed to irradiance stress. The damaged PSII centers remain as photoinactivated, without conducting electron transportation leading to slowdown of D1 degradation (pulse

chase and immunoblot experiments), which in turn slowed D1 replacement (Vasilikiotis et al., 1994).

Proteases

Proteases are involved in the removal of damaged D1 protein. The conformational changes in the damages D1 protein may act as signal for the removal of damaged D1 protein. The removal of damaged D1 protein takes place with the synthesis and insertion of new D1 protein (Aro et al., 1993).

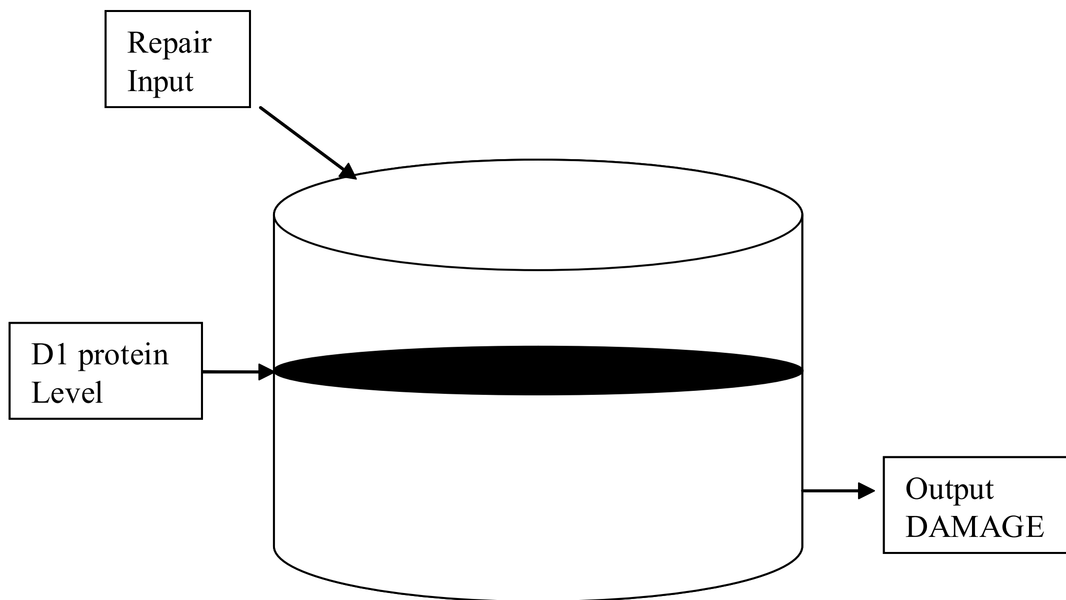


Figure 1. 4: Process of turnover of D1 protein

Zbigniew Krupa (1990), studied the recovery of photosynthesis on cyanobacterium *Anacystis nidulans* mutant strains, lacking one *psbA* gene of the three *psbA* genes. They reported the recovery from photoinhibition depends on the rate of repair of damaged D1 protein.

D1 protein degradation is a multi enzyme process, involving proteases, chaperonins GTP and ATP. In thylakoid membrane protein degradation, several families of proteases are involved. The FtsH holoprotein is thought to be a hexamer with ATP-dependent unfoldase activity mediated by the AAA domains present in integral part of thylakoid membrane is involved in disassembly of sub-units and degradation of the D1 protein. A second protease, the serine ATP-independent DegP, is predicted to be involved in degradation of other membrane proteins (Peter J. Nixon et al., 2004).

In *Synechocystis* sp. PCC6803, 62 proteases were thought genome and could in principal be, involved in the degradation and or removal of damaged D1 protein. In chloroplasts, DegP2 cleaves the stromally exposed side of the D1 protein between helices 4 and 5 of the D1 protein. In *Synechocystis* sp.PCC6803 a class of three serine type proteases belongs to DegP family were detected. These DegP proteins were predicted to play a role in the degradation of damaged D1 (Peter Nixon et al., 2004). The experiments conducted by Peter Nixon et al, 2004 using a *Synechocystis* sp.PCC6803 mutant lacking these three proteases does not discernibly effect the degradation of D1 protein, indicates the Deg P proteases play a minor if any role in D1 degradation.

In *Synechocystis* sp.PCC6803, the FtsH family of proteases contains four different members. Only two of them were proved to play an important role in the D1 repair process. FtsH protease is a membrane bound protease with a C-terminal leucine zipper domain, two trans membrane domains and an (AAA^+) ATPase binding domain along with a Zn^{2+} binding domain (Silva et al., 2003).

Silva et al, 2003 used a FtsH deletion mutant for their studies on the role of FtsH protease in the process of degradation of D1 protein. The repair process was inhibited in FtsH deletion mutant indicating FtsH is needed for the repair process. FtsH is involved in disassembly of damaged D1 protein and removal of damaged D1 proteins (Figure 1.5). The damaged PS II migrates from thylakoid to cytoplasmic membrane where it is synthesized. FtsH plays major role in presenting damaged D1 protein to degradation machinery (Silva et al., 2003).

One of the models for FtsH mediated degradation of D1 protein is based on the FtsH proteases in *E. coli* (Peter J. Nixon., 2004). According to this mechanism, the damaged D1 protein is degraded at Zn^{2+} center (Akiyama., 2002). FtsH protease acts on the exposed N-terminus of D1 for degradation; this process is followed by insertion of newly synthesised D1 into PS II (Figure 1.5). FtsH requires at least 20 amino acid length exposed N-terminus for its activity (Chiba et al., 2002).

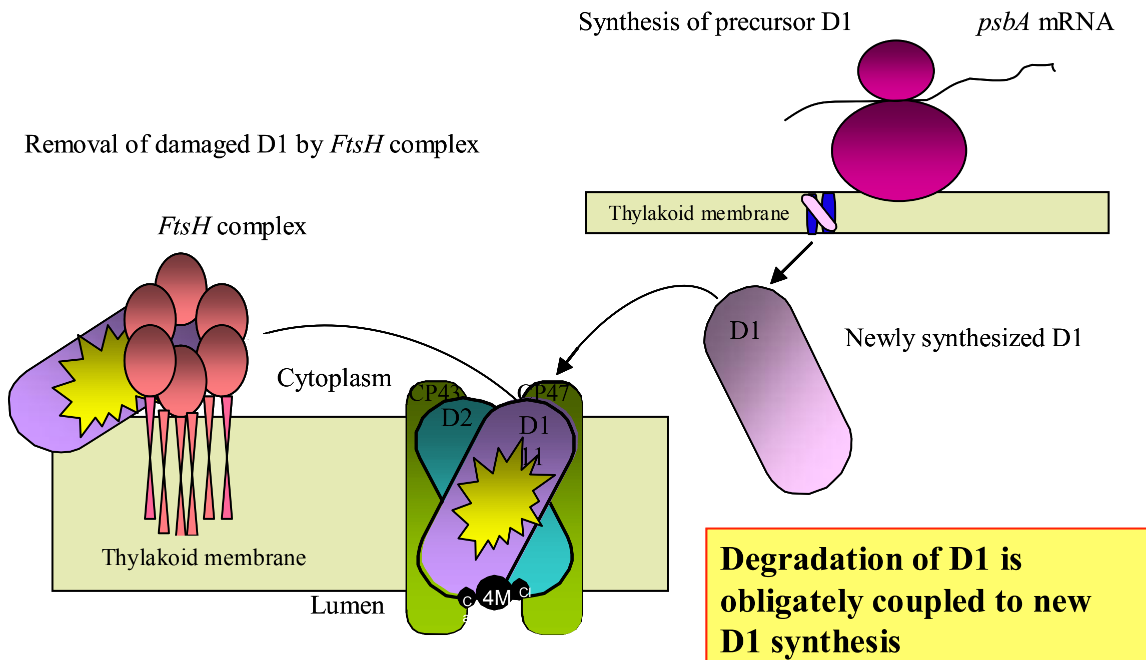


Figure 1.5: Action of FtsH in the removal and degradation of damaged D1 protein.

Based on the literature studies, it is evident that the mechanism of D1 protein turnover still needs to be elucidated. Our main interest was to identify the role of N-terminal, in the turnover of D1 protein. The experimental results caused a re-direction of the project towards identifying the role of transcript levels in the process of PS II repair.

Thesis Objectives

The repair of photosystem II is a highly dynamic process requiring the cooperative assembly of multiple proteins. I hypothesized that an ectopic expression system will allow better manipulation of the expression and mutagenesis of PSII proteins.

I chose to study the D1 protein as a model due to the importance of this protein.

Several technical objectives needed to be realized.

- Clone full length *psbA* gene in a vector system manipulation in *E. coli* and transferred to and expressed in *Synechocystis*.
    - This has never been achieved due to the toxicity of the major PSII proteins in *E. coli.*
    - I have succeeded for the first time in cloning full length psbA2 gene in *E. coli.*
    - I have succeeded in expressing D1 protein ectopically in non-native chromosomal location under artificial copper regulated PetE promoter.
- Technical objective:
    - Develop a mutagenic system that allows us to mutate the entire D1 protein including N-terminus (previous mutagenic systems allowed the mutation of C-terminus only).
    - Test expression of D1 from alternate chromosomal location and under artificial regulation (Cu-regulated promoter).
- Scientific objective:
    - Determine the factors controlling the repair of the PSII.

CHAPTER-II

Materials and Methods

Strains and plasmids

Synechocystis grows both photoautotrophycally and hotoheterotrophycally and can be easily transformed with high transformation efficiency, which is an important feature for genetic manipulation strategy (Matsunaga.,1990). In my thesis research I used the strain of wild type encoding the D1 protein *Synechocystis* PCC6803 and its deletion mutant, 4E-3, which lacks all three *psbA* genes (Debus et al., 1990). The 4E-3 strain was used as a host for transformation with mutant plasmids by homologous recombination, in order to prevent undesirable recombination between various genomic copies of *psbA* gene and the mutated *psbA* gene. Table 2.1 and Table 2.2, indicates the various strains and the recombinant plasmids used in our project.

Strains	Phenotype	Genotype	Reference
Wild Type	Autotrophic	Original	
WT*	Km^r	*PsbA2* in Original location, under 4E-3 background.	Chu et al.(1994)
4E-3	Heterotrophic Sm^r, Em^r,Cm^r	All three *psbA* genes were deleated leaving behind a part of N-terminus.	Debus R J (1990)
MK1	Autotrophic $Spec^r$	*psbA2* in different location under under 4E-3 background. .	Present study
MK2	$Spec^r$	*psbA2*+promoter in different location under 4E-3 background.	Present study

Table 2.1: *Synechocystis* strains used in the project, trophic and antibiotic phenotypes and the location of *psbA2* gene in these strains.

E. coli strains	Genotype	Reference
XL1-Blue Competent cells	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac. (Genes listed signify mutant alleles.)	Stratagene.
Plasmids		
pPETE	Low copy number PBR322 derivative. having copper promoter	Hong wang, unpublished
pRD1031	High copy number plasmid with ampicillin resistant cassette.	
MK1	*psbA2* gene in pPETE vector	Current Study
MK2	*psbA2* gene and *psbA2* promoter in pPETE vector	Current Study

Table 2.2: *E. coli* strains and recombinant plasmids used for construction of strains.

Phenotype	Selection	*Synechocystis*		*E. coli*
		Initial	Final	
Kmr	Kanamycin	5 µg/ml	50 µg/ml	50 µg/ml
Emr	Erythromycin	1µg/ml		750 µg/ml
Specr	Spectinomycin	5 µg/ml	20 µg/ml	50 µg/ml
Ampr	Ampicillin	1 µg/ml		60-125 µg/ml

Table 2.3: Working concentrations of different antibiotics used in this study for cultures growth and transformations for selection.

Growth and Culture conditions

All the *Synechocystis* strains were grown under illumination from white fluorescent lamps (50 µmol m^{-2} s^{-1}) at 32 °C. Mid-log phase cultures were used for experiments.

Plate culture

Synechocystis cells require mineral medium called BG-11(Rippka ., 1979) for their growth. Cells were initially streaked onto BG-11 agar plates with required antibiotic selection according to Table-2.3. These plates were incubated under approximately 50 µmol m^{-2} s^{-1} light intensity, at 32 °C.

Liquid culture

Under sterile conditions a loop full of cells from plate medium was transferred to 100 ml liquid BG-11 medium with appropriate antibiotic concentration into a 250 ml Erlenmeyer flask (autoclaved 24 hours before inoculation). These cultures were incubated in a 250 rpm rotary shaker under 50 µmol m^{-2} s^{-1} light intensity at 32 °C. Minimum time period of one week is required for the culture to attain the mid log phase when starting liquid culture from solid medium.

CO_2 Bubbling culture

The sample from 100 ml shaking culture was used to inoculate a 600 ml BG-11 culture in a 1 liter culture bottle. The culture was connected to bubbling apparatus (air enriched with 3% CO_2) in a water tank at a temp of 30ºC as

represented in Figure 2.1. The cultures were then allowed to grow until reaching mid to late log phase under low light (photon flux density 15-20 μmol $m^{-2} s^{-1}$) with constant CO_2 bubbling. The optical density of the culture at 750 nm (OD_{750}) was determined by using Shimadzu, uv-visible scanning spectrophotometer. The cultures were used for the experiments when the culture reached an OD_{750} in the range of 0.7-0.1.

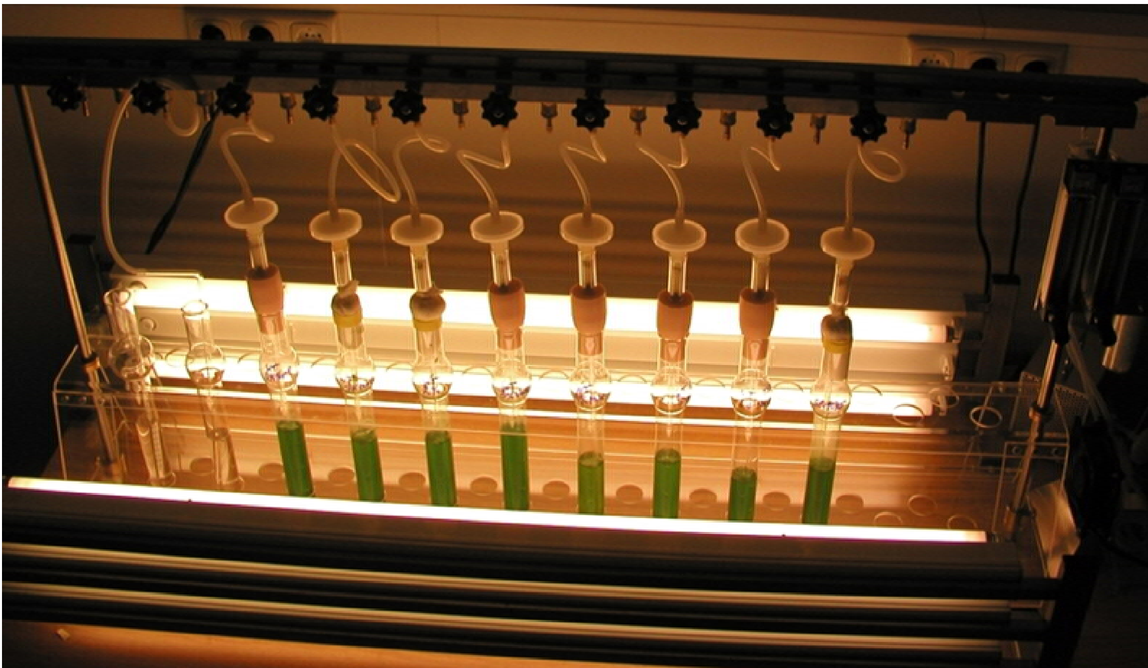


Figure 2.1: The actual set up of *Synechocystis* CO_2 bubbling culture

Construction of mutant strains

Cloning strategy

For construction of mutant *psbA2* plasmids, a low copy number plasmid called Ppete (Dr. Hong wang, unpublished data) was used. Initially pPETE vector contained the *patA* gene (Figure 3.1). The Ppete vector in linear form was obtained by restriction digestion of pPETE -*patA* plasmid with *SapI* Enzyme (New England Biolabs).

pPETE -*patA* vector was digested with *SapI* using following conditions: 48 µl of double distilled water, 8 µl of 10x Buffer (NE Buffer 4), 16 µg Ppete-*patA*, 8 µl of SapI (New England Biolabs) in a total reaction volume of 80 µl. The reaction mixture was vortexed and incubated for 4 hours in a 37°c water bath. This restriction digestion resulted in the removal of the *patA* insert from the vector Ppete.

Purification of linerized Ppete vector by preparative gel Electrophoresis

SapI digested DNA was used to separate 6.5kb Ppete Vector by using 0.8% agarose gel in TAE (Sambrook et al., 1989). The bands were excised from the gel under UV light and the excised band with DNA was transferred to a sterile microcentrifuge tube. Ppete vector DNA was extracted from the gel by using Qiagen Qiaex II gel extraction kit (Qiagen, 27220 Turnberry Lane Suite 200 Valencia, CA 91355), according to the user manual.

Primer design for *psbA2* gene

Engineered primers were used for the amplification of *psbA2* gene along with flanking regions that introduce restriction enzyme sites for the type II restriction endonuclease *SapI*. An overhang sequence AATTAAGCTCTTCC was added to the primers as flanking sequence to amplify *psbA2* gene along with these flaking regions for further insertion of *psbA2* into *Synechocystis* chromosome into neutral site. Each primer was designed with 50% GC content.

Primer	Sequence
psbA2 forward	5^1 AATTAAGCTCTTCCATGACAACGACTCTCCAACAGC 3^1
	3^1 TTAATTCGAGAAGGTACTGTTGCTGAGAGGTTGTCG 5^1
psbA2 reverse	5^1 GCTCCTGCTGTCAACGGTTAACGAAGAGCATATAT 3^1
	3^1 CGAGGACGACAGTTGCCAATTGCTTCTCGTATATA 5^1
*psbA2*_promoter forward	5^1 AATTAAGCTCTTCCATGTTAAGAGTAATGGCGTGCAAGG 3^1
	3^1 TTAATTCGAGAAGGTACAATTCTCATTACCGCACGTTCC 5^1
*psbA2*_promoter reverse	5^1 GCTCCTGCTGTCAACGGTTAACGAAGAGCATATAT 3^1
	3^1 CGAGGACGACAGTTGCCAATTGCTTCTCGTATATA 5^1
AATTAAGCTCTTCCATG	This is the flanking sequence added for each primer.

Table 2.4: Sequence of *psbA2* primers and the flanking region used for amplification of *psbA2* gene for construction of mutants. The *psbA2* gene was amplified along with engineered flanking region on either side, for insertion of the construct into neutral site of 4E-3 chromosome through homologous recombination (Figure 3.2).

Amplification of *psbA2* gene by PCR

PsbA2 gene was amplified from *Synechocystis* PCC6803 chromosome by PCR. The total reaction volume used was 100 µl and the reaction was composed of 70.2 µl double distilled water, 10 µl of 10X PCR buffer (Invitrogen), 2µl of 50mM $MgSO_4$ (Invitrogen), 1.5 µl of 10 mM dNTP's, 5 µl of 20 µM primer Pair (Integrated DNA Technologies) (Table 4), 0.5 µl of 500 µg/ml template, 10 µl of 10x Pfx enhancer (Invitrogen) , 0.8 µl of Pfx polymerase 2.5 U/ml (Invitrogen).

PCR program parameters	
Initial Denaturation	94°C for 2 minutes
Denaturation:	94°C for 30 seconds
Annealing	62°C for 45 Seconds,
Extension	68°C for 3 minutes x 25 Cycles, 68°Cfor 20 min
Hold	15°C

Table 2.5: PCR program parameters for amplification of *psbA2*

Purification of PCR amplified product

PCR amplified *psbA2* was purified by Qiagen PCR Purification kit as per the user manual (27220 Turnberry Lane Suite 200 Valencia, CA 91355).

Ethanol Precipitation

Ethanol precipitation process is followed for the differential precipitation of DNA for better purification of nucleic acids. The nucleic acid to be purified was mixed with 1/4 volume of 7.5 M ammonium acetate (1/4 of total nucleic acid volume) and 2.5 volumes of 95% absolute ethanol (2.5 volumes of the total nucleic acid volume) and incubated at -20°C overnight. The treated samples were centrifuged 12,000 g for 10 minutes to pellet the DNA. The obtained pellet was treated with 70% ethanol wash. The pellet was air dried and re-hydrated in 20 µl distilled water.

Ligation

Ligation was performed in 20 µl reaction volume by using vector and insert in the ration of 2:1 (µg). The master mix was made up with 2 µl of 10X Ligation Buffer, 1 µl of T4 DNA Ligase (promega). The reaction volume was made up to 20 µl with distilled water and was incubated at 14°c for 18 hours. The ligation mixture obtained from this process was ethanol precipitated and was re-hydrated in 4 µl distilled water. One microliter of this ligation product was used for transformation into *E. coli*.

Transformation into XL-1 Blue Super Competent Cells

XL-1 blue super competent cells (Stratagene, La Jolla, CA) were used to according to user manual of Stratagene. LB plates with 30 µg spectinomycin plates were used for selection of the transformants. The efficiency of obtained clones with the correct insert was found to be 80% in this case.

Note: It was found to be important to use fresh plates containing spectinomycin for transformation experiments because spectinomycin degrades quickly and therefore plates older than one week do not show effective selection.

Isolation of plasmid from the colonies

Single colonies were chosen from the transformation plates and inoculated into 2ml LB liquid medium supplemented with 30 µg spectinomycin. These cultures were incubated in a rotary shaker at 250 rpm at 37°C overnight. Plasmid DNA was isolated from these cultures by alkaline lysis (Sambrook,.1989).

DNA Sequence

Confirmation of cloned strains was determined by restriction digestion and DNA sequencing. Automated DNA sequencing was performed by the recombinant DNA and protein resource facility housed in the Biochemistry department at Oklahoma State University.

Transformation of *Synechocystis*

The cells were harvested and resuspended in BG-11 at a density of approximately 10^9 cells/ml. Five to ten micrograms of DNA was used to transform 300 µl of the *Synechocystis* cells and glucose was added to the culture to a final concentration of 15 mM. The transformation mixture was incubated under 50 µ mol m^{-2} s^{-1} fluorescent lamp in a 250 rpm shaking rotary culture with gentle agitation in 12.0ml falcon tubes at 32°c for 4-6 hours to allow DNA uptake. Later, 3 ml of BG-11 liquid medium was added to the transformation mixture in 125 ml flasks which were incubated for 24 hours under *Synechocystis* growth conditions. The 24 hour period allows the uptake and integration of the transformed DNA into the chromosomes and will provide time for the expression of the selection marker.

The transformation mixture was then plated on BG11 plates containing appropriate antibiotic according to Table 3. The transformation results were visualized in 7 to 10 days after transformation. In order to obtain complete segregation of the mutant, the transformed colony was streaked two three times on increasing concentration of antibiotic before proceeding with physiological characterization.

Extraction of DNA *Synechocystis* cells

A thick loop-full of cyanobacterial culture cells were resuspended in 200 µl of TE (Sambrook et al., 1989) in a microcentrifuge tube and 300 µl of 1:1 mix of TE glass beads was added to the cells and they were lysed by using bead beater for 30 seconds. The glass beads were allowed to settle to bottom of the

tube and aqueous phase (containing the DNA) was transferred into a new micro centrifuge tube.100 μl of TE was added to the glass beads and mixed well and the process of collecting aqueous phase was repeated. The supernatant from these two steps was mixed and was centrifuged at 12,000 g for ten minutes at room temperature. Approximately 200-300 μl of the supernatant containing DNA was collected into a new micro centrifuge tube. 200 μl of TES-equilibrated phenol was added and centrifuged for five minutes at 12,000 g. Further, the supernatant was transferred into a new tube and it was extracted with one volume of chloroform to remove traces of phenol. The isolated supernatant was ethanol precipitated and the resulted pellet was re-suspended in 20 μl of TE.

Light experiments

The cultures from CO_2 bubbling bottles at an O.D 750nm of 0.7-0.1 were used for the photoinhibition and recovery experiments with light exposure in 30°c water bath (Figure 2.1).

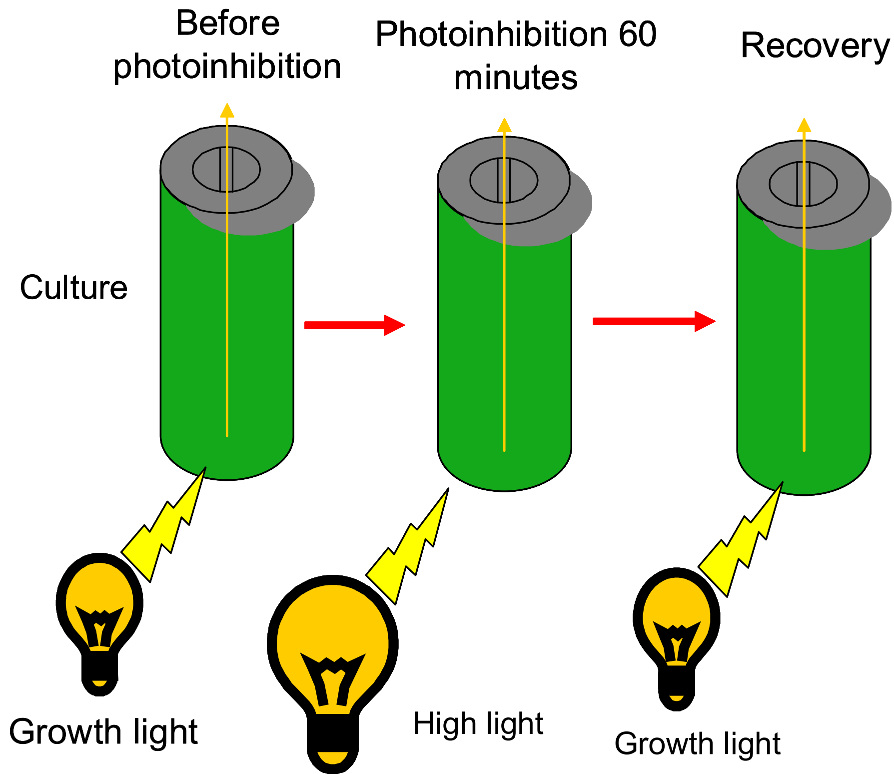


Figure 2.2: Experimental set up for photoinhibition and recovery experiments

The experiment was set up in a pyrex tube or culture bottle with CO2 bubbling and light exposure as represented in Figure 2.2. WT and WT* were exposed to 1000 μmol m^{-2} s^{-1} and MK1 and MK2 were exposed to 800 μmol m^{-2} s^{-1} light intensity. Samples were collected at time points of 0, 15, 30, 60 minutes of photoinhibition and variable fluorescence measurements was recorded for the collected culture. After 60 min of photoinhibition, the tubes were transferred from high light to growth light (20 μmol m^{-2} s^{-1}). Samples were collected at time points of 0, 15, 30, 60, 90 minutes etc, every 30 minutes from recovery tubes until 3 and half hours and variable fluorescence measurements was recorded for the collected culture.

Chlorophyll fluorescence measurements

Chlorophyll flourescent measurements are important to know the photosynthetic performance of strains and the extent of PSII damage to high-light intensity. Measurements of chlorophyll a fluorescence were conducted with a PAM-200 Walz (Effeltrich, Germany) pulse amplitude-modulation flourometer.

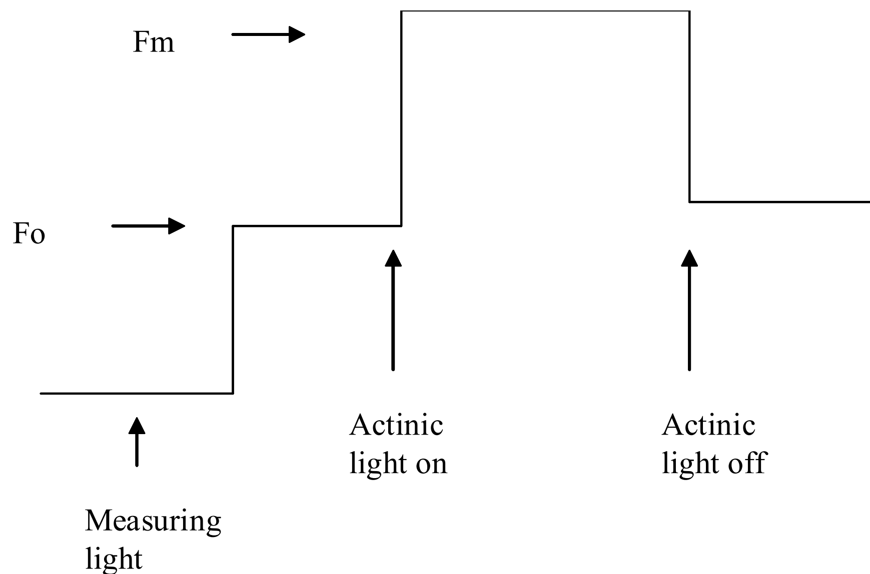


Figure 2.3: Chlorophyll florescence measurement

The experiment was conducted at 1.6 or 100 kHz. Initial florescence (Fo) was induced by weak measuring light. Measuring light is a weak probe of florescence yield. After application of the saturating actinic flash the fluorescence level increased from Fo to Fm (maximum fluorescence) indicating the reduction of plastoquinone PQ_A (Figure 2.3). The actinic flash was used to reduce completely the PSII acceptor side PQ_A and to measure the maximum fluorescence yield (Fm).

The variable fluorescence (Fv) was calculated using the formula,

$$Fv = \frac{Fm/Fo}{Fo}$$

Potential yield of the photochemical reaction of PSII was measured by the ratio of Fv/Fm.

The measurement of variable fluorescence using chemicals is called $F_{HA\text{-}QA}$. An oxygen evolution measurement of chlorophyll fluorescence gives a measurement of active D1 proteins. The active D1 proteins was estimated by measuring the difference between initial fluorescence (Fo) and maximum fluorescence (Fm). Fm was measured by dark incubation of cells for 5 minutes with p-benzoquinone and potassium ferricyanide followed by 1minute dark incubation in the presence of DCMU and 20 mM hydroxylamine (Procedure adopted from Debus R.j, Chu et al., 1994).

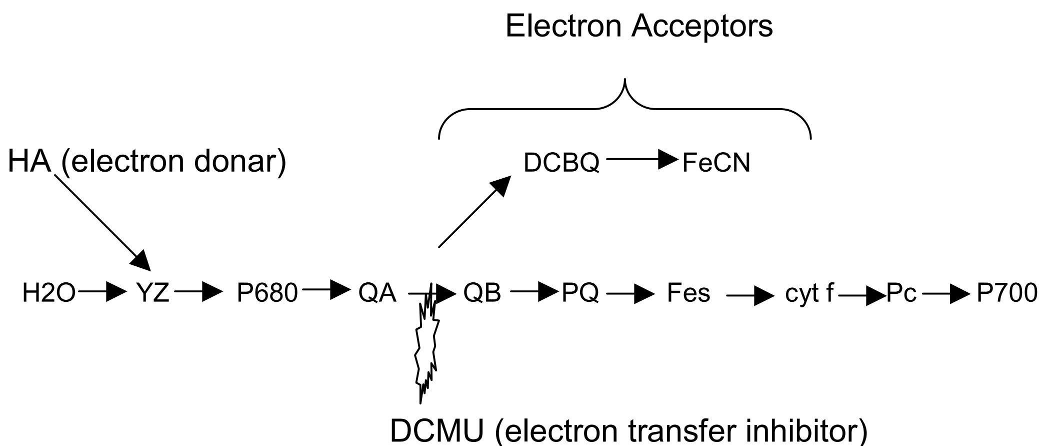


Figure 2.4: Action of electron transfer inhibitor DCMU, Electron acceptors DCBQ and Fecn, Electron donor HA on electron transport in $F_{HA\text{-}QA}$ measurements.

Technique for growing Synechocystis cultures for RNA isolation

RNA was isolated from four different strains of *Synechocystis* at 4 different time points. The strains used for RNA isolation were WT, WT*, MK1 and MK2. The culture from CO_2 bubbling bottles at an O.D 750nmof 0.5-0.7 were used for RNA isolation.

Harvesting the cells and extraction of RNA

Cell Harvesting

For harvesting cells for RNA isolation 200 ml of culture having an O D 750 nm of 0.5-0.7 was transferred into 200 ml centrifuge bottle (phenol-resistant) pre-filled with 20 ml of 20 X Stop solutions and centrifuged at 4ºc for 10 min at 7600xg. The supernatant was resuspended in 600 ul of suspension buffer (prepared by mixing 0.3 M sucrose and 10mM Sodium acetate pH 4.5), the resuspended cells were then transferred into microcentrifuge tubes, and cells were pelleted by centrifugation at 8,000 xg for 1 min at room temperature and the cell pellet was frozen in liquid nitrogen.

Isolation of RNA

The cells were thawed on ice and 38 µl 0.5 M EDTA at pH 8.0, 320 µl of cell suspension buffer (prepared by mixing 0.3 M sucrose and 10 mM Sodium acetate pH 4.5) were added to resuspend the cells. To the resuspended cells 340 µl of 100/9 mM sodium acetate (prepared by mixing 1 part 1 M sodium acetate stock with 8 parts nuclease free water), 38 µl of 20% SDS were added and mixed well. The cells were then incubated at 65 ºc for 5-10 min and 700 ul

hot acidic phenol (65ºC) was added. The contents were mixed well and Incubated at 65 ºc for 5 minutes. Further the cells were cooled at -80 ºc for 45 seconds and centrifugation at 8,000 xg for 5 min at room temperature to separate aqueous phase from phenol. The aqueous phage was extracted with equal volumes of hot acidic phenol one more time to remove proteins. The obtained aqueous phage was extracted once again with Room temperature 1:1 phenol/chloroform solution. To the supernatant containing the RNA, 1/4 volume of 10M LiCl, 2.5 volumes of absolute alcohol was added and was incubate at -20 °C for 50-60 min. The RNA was pelleted at full speed for 10 min and Washed with 70% ethanol. The RNA pellet was air dried and dissolved in 50 µl of nuclease free water and stored it at -80 °c.

RNA cleanup using "Ambion DNA freeTM kit"

RNA can be purified from genomic DNA contamination by using Ambion DNA free kit (Ambion Diagnostics). 10 µg of RNA in a 50 µl reaction can be purified. 0.1 volume of 10 X DNase I buffer and 1µl rDNase I was added to the RNA and incubated at 37 °c for 30 minutes. 0.1 volume of DNase inactivation reagent was further added to the mixture and was incubated for 2 minutes at room temperature. The reaction mixture was centrifuged at 12,000 xg and the supernatant containing RNA was removed and ethanol precipitated. The pellet was dissolved in 20µl nuclease free water and 0.8% agarose gel was run to check the elimination of genomic DNA contamination.

cDNA SYNTHESIS

Probe preparation

The reaction mixture consisted of 10 µg of RNA, 8 µg of Genisphere Array 900MPX random primers, with a final volume made to 20 µl with sterile distilled water. The reaction mixture was incubated at 80 ºc for 10 min followed by chilling treatment on ice for 2-3 min.

2XRT Master mix was prepared in a microcentrifuge tube according to the Table-2.6.

2X RT Reaction Master Mix	*2.5 rxns*	*4.5 rxns*	*6.5 rxns*	*8.5 rxns*
0.1M DTT(Invitrogen)	12.5	22.5	32.5	42.5
5X FSB (Invitrogen)	25	45	65	85
50X aadNTP mix (See above) (Invitrogen)	2.5	4.5	6.5	8.5
DEPC treated H_20 (Invitrogen)	17.5	31.5	45.5	59.5
SSII enzyme(Invitrogen)	5	9	13	17
Total volume	62.5	112.5	162.5	212.5

Table 2.6: Representing the amount of components for preparation of 2XRT Master mix for cDNA synthesis**.**

25 µl of RT Master mix (Table 2.6)was added to each tube and was incubated at 42 ºc for 3 hours. Hot alkali hydrolysis of RNA template was performed by adding 5 µl of 50 mM EDTA and 2.5 µl 10 N NaOH to the reaction mixture by incubating at 65 °c for 20 min. This is followed by addition of 5 µl 5 M acetic acid to neutralize the reaction mixture. YM-30 clean up columns was used to concentrate the cDNA (Procedure adopted from Dr.Patricia Ayoubi, 360A NRC-D. Oklahoma State University).

The reservoir membrane was pre washed by adding 100 µl (nuclease free) ddH_2O by centrifuging at 12,000 xg for 5 min. 450 µl (nuclease free) ddH_2O was added to each sample of cDNA mixtures and transferred to appropriately labeled Micron YM-30 sample columns. The cDNA was concentrated by centrifuging the columns with cDNA mixture for 20 min at 12,000 xg. These wash steps were repeated three more times with 450 µl (nuclease free) ddH_2O. After the final wash step 10 µl of (nuclease free) ddH_2O was used to elute the cDNA. The obtained cDNA samples were stored at -80 °c. The cDNA concentration was measured using a spectrophotometer. "An OD of 1 corresponds to ~50 µg/ml for double stranded DNA, 40 µg/ml for single stranded DNA and RNA and ~33 µg/ml for single stranded oligonucleotides" (Molecular cloning, Third edition, Volume3, Appendix A8.20, Joseph Sambrook and David Russel).

Real Time PCR

Two step real time PCR was performed to quantify the transcript levels in different strains. The primary step involves preparation of cDNA from RNA by using reverse transcriptase (super script II, Invitrogen), and the secondary step

involves using the cDNA prepared in step one for quantification in real time PCR. The $^{\Delta\Delta}$Ct method of relative quantification method was used with SYBR Green, to estimate the transcript levels of *psbA* genes.

The cDNA samples used for real time PCR

Steady state (0-time point) culture of control strains WT, WT* and mutants MK1, MK2

Recovery 60 minute time point samples of control strains WT, WT* and mutant strains MK1, MK2.

Oligonucleotide primer design

Primer	Sequence	Tm
psbA2 forward	5' TAA CCT CCT CCT TGG TGG GTG AAA 3' 3' ATT GGA GGA GGA ACC ACC CAC TTT 5'	60.3°c
psbA2 reverse	5' AGA TGA ACC GAC CAA AGT AGC CGT 3' 3' TCT ACT TGG CTG GTT TCA TCG CCA 5'	60.3°c
rpnB forward	5' TAA GGG TGC AAA GGT GCG GTA AGA 3' 5' ATT CCC ACG TTT CCA CGC CAT TCT 3'	60.5°c
rpnB reverse	5' TTC CTC AAG GGG TTC CAC CAA TCA 3' 3^{1} AAG GAG TTC CCC AAG GTG GTT AGT 5^{1}	60.8°c

Table 2.7: Real time PCR primers

QRT-PCR assay

The stock cDNA of the six samples were used for preparation of the working solutions with 20 ng/µl concentration. The PCR reactions were conducted in the 96 well plates (Applied biosystems), in 25 µl volume containing 50 ng of the template, 12.5 µl of SYBR Green I ® master mix (Applied biosystems, part no: 4309155), 1 µl of PCR primer pair (10 µm), and the final volume was made up with water.

BIO-RAD real time PCR machine parameters

Denaturation: 95 °c for 10 minutes followed by 95°C for 15 seconds

Annealing: 55.0 ºc for 30 seconds

Extension: 72.0 ºc for 30 seconds.

40 cycles until step 2, with 4 ºc hold at the end.

At the end of the experiment, the reports were stored for analysis by setting up cycle threshold (Ct) manually to a point, where all the samples were at log phage of the cycle.

Standard curve

For standard curve 6 different 10 fold serial dilutions of WT cDNA was prepared according to Figure 2.5 and *psbA2* and control primer *Rmpb* were used.

Dilution series:

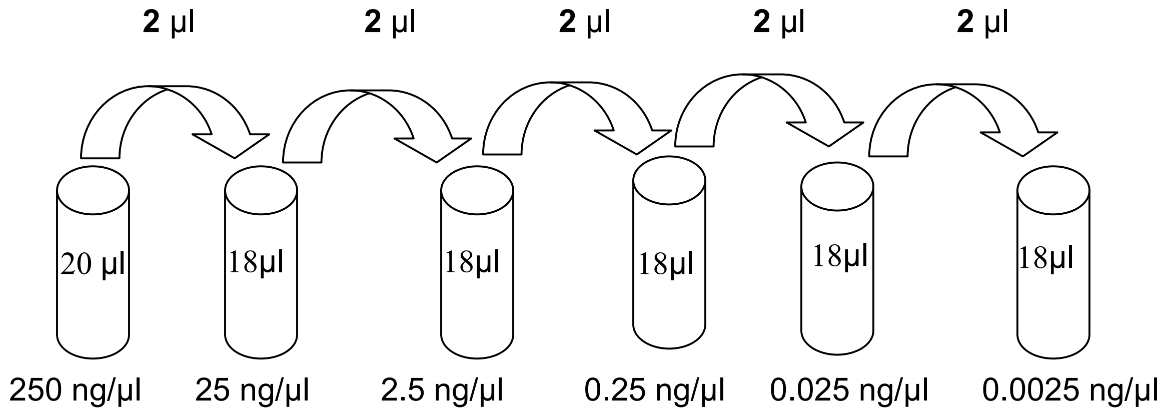


Figure 2.5: Flow chart showing the dilution series for standard curve method

4 µl of template was used from each dilution series in 25 µl volume for standard curve method. Standard curve method is important to know PCR efficiency at different concentrations of cDNA and to pick right concentration.

ΔΔCt method of relative quantification

Ct is the cycle number at which the (log phage of cycle) set threshold is reached. The fold change in the transcript levels of mutants were calculated by comparing ΔCT values using the $2^{-\Delta\Delta CT}$ method. The $^{\Delta\Delta}$Ct is obtained by ($^{\Delta}$Ct of target gene- $^{\Delta}$Ct of control gene). Relative Ct is obtained by comparing Ct values of WT*, MK2 and MK1 to Ct value of WT (Ct of target -Ct value of X gene= Relative Ct of X gene in comparison to target gene). If the relative Ct is positive it represents that gene is up regulated compared to target gene, and if the relative Ct value is negative it represents the gene is down regulated compared to target gene.

Growth curve

Time course growth measurements were taken for WT, WT*, MK1 and MK2 strains. The cultures were initially diluted to an OD 750nm of 0.1, in 250ml BG-11 medium. The cultures were grown under 20 μmol m^{-2} s^{-1} photon flux intensity with CO_2 bubbling condition. The OD of these cultures was measured every 6 hours. The doubling time of all the strains was calculated.

Site-Directed Mutagenesis:

Stratagene mutagenic kit (Stratagene, La Jolla, CA 92037) was used to convert Cysteine-18 to Alanine.

Primer design:

Mutagenic primers were designed with the insertion of desired mutation and a null restriction site at the region of mutations for conformation of mutations.

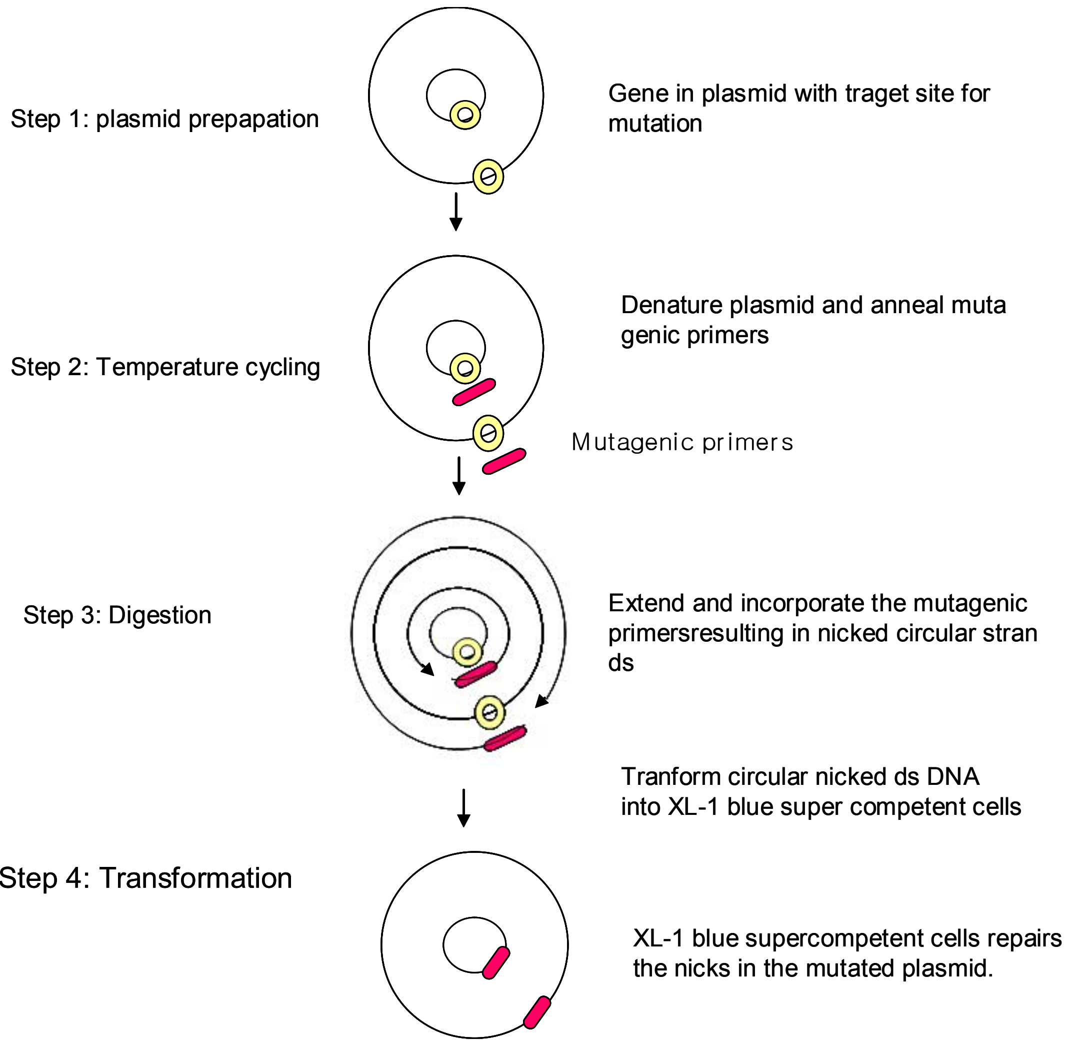
Cysteine-18 to Alanine mutation primers	
Forward primer	5^1 CTTGTGGGAACAGTTTGCGCAGTGGGTGACCTC 3^1
Reverse primer	5^1 GAGGTCACCCACTGCGCAAACTGTTCCCACAAG 3^1

Table 2.8: Cystene-18 to Alanine mutation primers.

The constructed mutagenic primers were used for the mutagenesis of the plasmid in the thrmemal cycler(Table 2.9).

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length*

Table 2.9: PCR parameters for site directed mutagenesis.



Source: QuikChange® Site-Directed Mutagenesis Kit

Figure 2.6: Site directed mutagenesis method as per Stratagene site directed mutagenic kit protocol.

Confirmation of mutations:

The obtained mutagenic plasmid was restriction digested with the desired restriction enzyme and was sequenced to confirm the mutations in frame.

CHAPTER-III

Ectopic expression of the D1 protein

Introduction

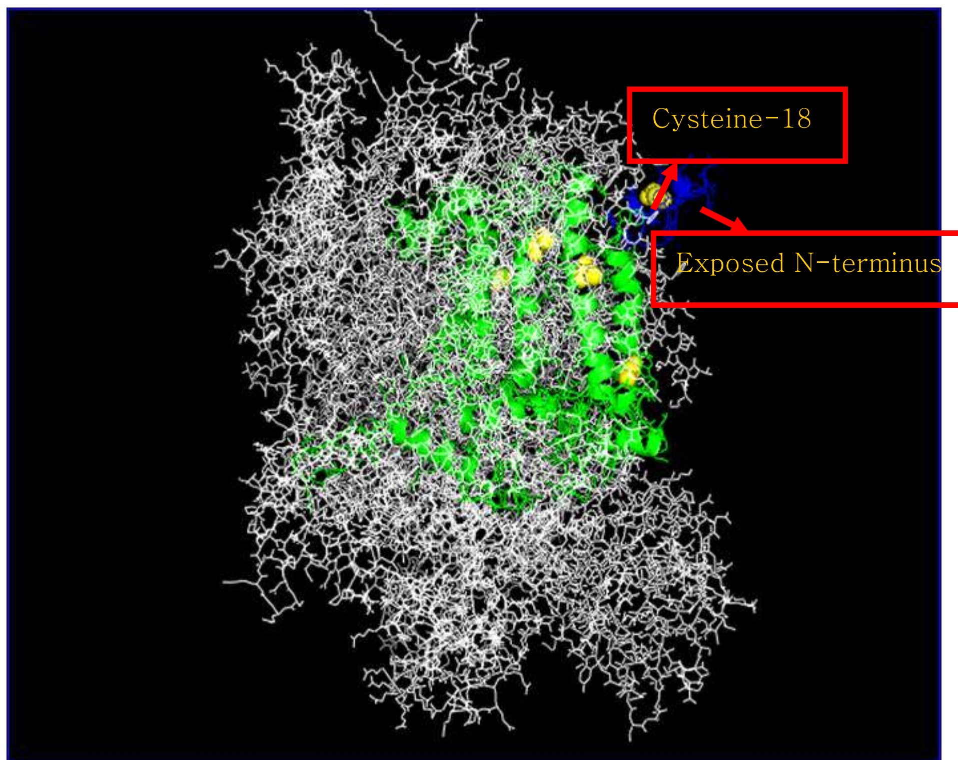
To study the structural and functional characteristics of the D1 protein, the expression of the full-length *psbA* gene is required. Repeated efforts of scientists attempting to clone the full-length *psbA* gene along with its native promoter have met with failure, although the literature does not record these unsuccessful efforts (see Debus et al., 1990). In order to permit the expression of wild type and mutant forms of the highly expressed *psbA2* gene in *Synechocystis* sp. PCC6803, a system involving a suitable recipient strain along with a non-toxic cloning strategy needed to be developed to study PSII function in this important experimental model. This task was most successfully accomplished by the construction of a *psbA* deletion strain called '4E-3', in which all three of the native *psbA* genes were either fully or partially replaced by different antibiotic resistance cassettes. This strain then would serve as a recipient of altered forms of the *psbA2* gene, which had been modified to study the function of the D1 protein. To facilitate cloning of the otherwise toxic *psbA* gene, a segment of the *psbA2* locus encompassing the promoter region and a small portion of *psbA2* gene N-terminus was omitted from the vector. In the meantime, the 4E-3 strain was

constructed so that one of the *psbA* genes was only partially replace: a slightly larger portion of the *psbA2* locus covering the promoter and N-terminal segment of *psbA2* gene remained at its original location (Debus et al., 1990). This system allowed the researchers to clone and mutationally characterize about 85% of D1 protein. However, alternative system would be required to study the N-terminal portion of the D1 protein. While such a D1 N-terminal mutagenesis system was successfully constructed, it had the opposite problem of not being suitable for analysis of the C-terminal portion of the D1 protein.

The technical objective of my research was to construct a mutagenic system that allows us to mutate entire D1 protein including the N-terminus and to test expression of D1 from alternate chromosomal location and under artificial regulation (Cu-regulated promoter). The technical objective was to address my hypothesis that the exposed N-terminus or N-terminal cysteine-18 is required for the action of FtsH protease in the process of removal of damaged D1 contained within the photosystem II during the repair process. This hypothesis is based on the studies of Chiba et al in 2002 on investigating the action of FtsH protease in *E. coli*. They found that FtsH needs exposed N-terminus for its protease action. The protease action of FtsH in *Synechocystis* sp. PCC 6803 has proved by Peter J. Nixon., 2004. But the mechanism of action of FtsH protease is not known. Based on above findings I hypothesised that the exposed N-terminus or N-terminal cysteine-18 of D1 protein part of mechanism of D1 turnover. Additionally, I hope that an alternative *psbA* expression system can be developed

to allow the expression of the D1 protein artificially since such a strain could, in principal, be very useful for the analysis of PSII repair.

I hypothesized that cloning of the full-length *psbA* gene would be possible by reducing its expression when it is being propagated in an *E. coli* host and that this could be accomplished by utilizing a low copy-number vector and by not including its native promoter which had been shown to be highly active in *E. coli.* In this chapter, I will discuss and describe the steps taken to test my hypothesis. I will show how such a vector, the pPETE plasmid previously constructed in our lab, can be used to clone full length *psbA2* gene in *E. coli*. This cloning process is described in detail in results section.



Source: Athina Zouni et al *Nature* **409**, 739-743 (8 February 2001)

Figure 3.1: Exposed N-terminus and cysteine-18 of D1 protein from photosystem II.

Why cysteine-18?

Cysteine-18 present at the N-terminus of D1 protein was thought to be involved in the removal of damaged D1 protein as cysteine-18 is conserved in all photosynthetic organisms except an organism *Gleobacter,* which is thought to represent an ancient cyanobacterial lineage and appears to be more primitive on the basis of rRNA sequence and ultra structural characteristics such as the location of the photosynthetic apparatus on the cytoplasmic membrane and the complete absence of a thylakoid membrane system (Rippka et al.,1974). I speculate, although I do not test the hypothesis that *Gleobacter* does not possess the highly efficient PSII repair mechanism found in more modern strains. Consequently, *Gleobacter* does not utilize a cysteine at the N-terminus since it lacks the more advanced repair mechanism.

Figure 3.2: Gene doc alignment of *psbA2* gene in different photosynthetic organisms showing conserved cysteine-18 in all organisms except an ancient organism gleobacter, in which cysteine -18 was replaced by alanine.

To address my hypothesis two *psbA2* mutants were constructed. These mutant strains allow expressing and mutating entire D1 protein including N-terminus.

Results and Discussion

Construction of *psbA* mutant strain:

Vector construction:

Using high copy-number plasmids such as pUC19 as cloning vehicles, genes encoding transmembrane PSII proteins were unable to be cloned as full length open-reading frames (Chisholm and Williams., 1988). This characteristic may be because *E. coli* recognizes the cyanobacterial promoter and the gene, when expressed, results in toxic products resulting in disruption of the *E. coli* membrane (Philbrick et al., 1988).

In order to create *psbA2* mutants with a system permitting manipulation of the entire gene, the *psbA2* gene had to be inserted into a vector capable of tolerating the full length gene in *E. coli*. A low copy number plasmid called Ppete, encoding a spectinomycin resistant cassette having transcription termination signals and is expressed under a Cu-regulated *petE* promoter was used. The promoter controls the expression of the plastocyanin gene in *Synechocystis* sp. PCC6803, and has been shown to be active in the mineral media, BG-11 that was used in this study (reference: Wang Postier and Burnap). Furthermore, the activity of the *petE* promoter is not influenced by changes in light intensity according to previous unpublished results in our laboratory (that will appear in the thesis work of my colleague, Rekha Nambudiri). Two *psbA2* mutants called MK1 and MK2 were constructed using this vector as explained in Figure 3.3. Fragments of DNA containing the entire *psbA2* gene amplified along with engineered non-native flanking regions containing the *Sap*I restriction site

were amplified using hot start PCR with *Synechocystis* chromosomal DNA as the template and chimeric primers containing the engineered *SapI* restriction site. The *SapI* restriction enzyme cleaves the target sequence outside of the recognition sequence leaving overhangs that are not important for recognition by the enzyme. The vector and PCR primers were designed so that ligation of the restricted PCR products and plasmid DNA produced compatible ends allowing only unidirectional cloning of the PCR product and ensured that the ligation of insert and vector produced a circular product with the *psbA2* gene in the desire direction. Once the amplified sequences were inserted into Ppete vector (low copy number) by ligation, they were used to transform *E. coli* using spectinomycin for selection of plasmid-bearing strains.

Confirmation of the desired plasmids was confirmed by restriction digestion of the isolated plasmids (details provided in the Chapter 2, Materials and Methods). It was also important to conduct sequence analysis of these plasmids, to confirm the insert was without mutation. I succeeded in cloning full-length *psbA2* gene into *E. coli*. This is the first time that the full length open reading frame has been cloned into *E. coli*. The success may be due to use of low copy number plasmid for cloning and the absence of the strong promoter normally upstream of the *psbA* gene. The confirmed plasmids were named as plasmid MK1 (pMK1) (Figure 3.4) and plasmid MK2 (pMK2) (Figure 3.5).

Construction of strain:

a)

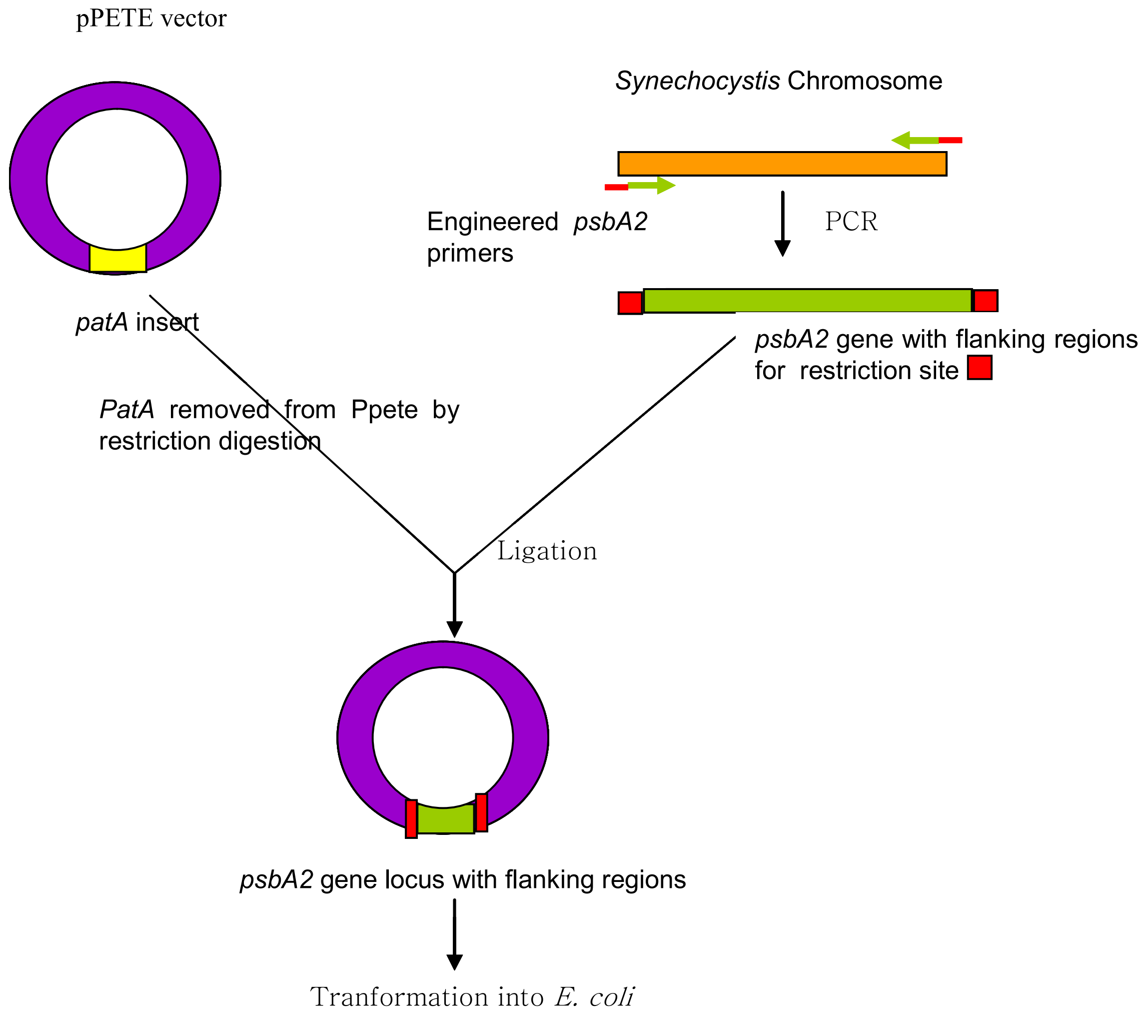


Figure 3.3:

a) Cloning strategy used to clone *psbA2* gene into Ppete vector. *psbA2* constructs were amplified from *Synechocystis* chromosome using *psbA2* primers with engineered DNA flanking sequence, which were able to amplify *psbA2* gene copy with addition of flanking DNA sequence on both sides (Table 2.4). The flanking regions are the DNA sequence regions for restriction digestion, to ligate the constructs into vector.

Constructs cloned

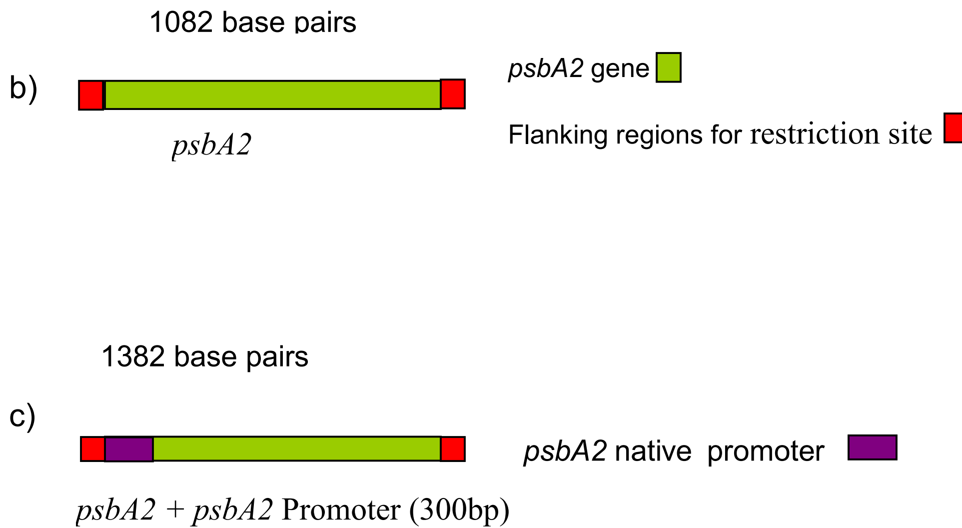


Figure 3.3:

b) Constructs cloned (Ectopic locus of MK1):1082 base pairs of (slr1311) *psbA2* gene sequence was amplified with flanking sequences on both sides of gene using *psbA2* primers with engineered restriction site DNA flanking sequence (Table 2.4)

C) Construct cloned (Ectopic locus of MK2): 1382 base pairs of (slr1311) *psbA2* gene with *psbA2* promoter sequence was amplified with flanking sequences on both sides of gene using *psbA2* primers with engineered restriction site DNA flanking sequence (Table 2.4).

Plamid MK1 (pMK1)

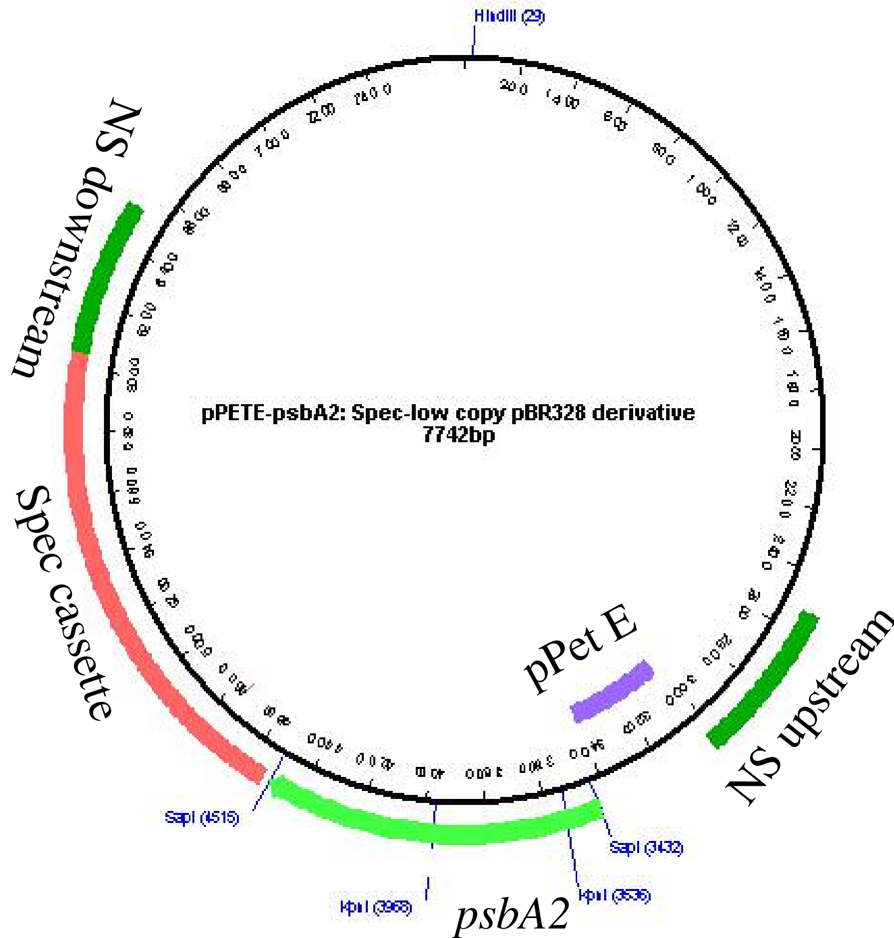


Figure 3.4: Plasmid map of MK1 indicating locations of different fragment positions in the map. For ligation of vector and insert SapI restriction enzyme was used. The vector contains upstream and downstream neutral site regions for transformation of the construct into *Synechocystis* chromosome through homologous recombination. Neutral sites are the regions homologous to the regions of recombination in *Synechocystis* chromosome for introduction of constructs into the host chromosome. pPet E is the copper regulated promoter present upstream to the *psbA2*, Spec cassette present downstream to the *psbA2* gene with transcription termination region in it. For the confirmation of construct, restriction digestion was used. KpnI enzyme sites are present with in the *psbA2* gene and Hind III is present is present in the pPETE vector.

Plamid MK2 (pMK2)

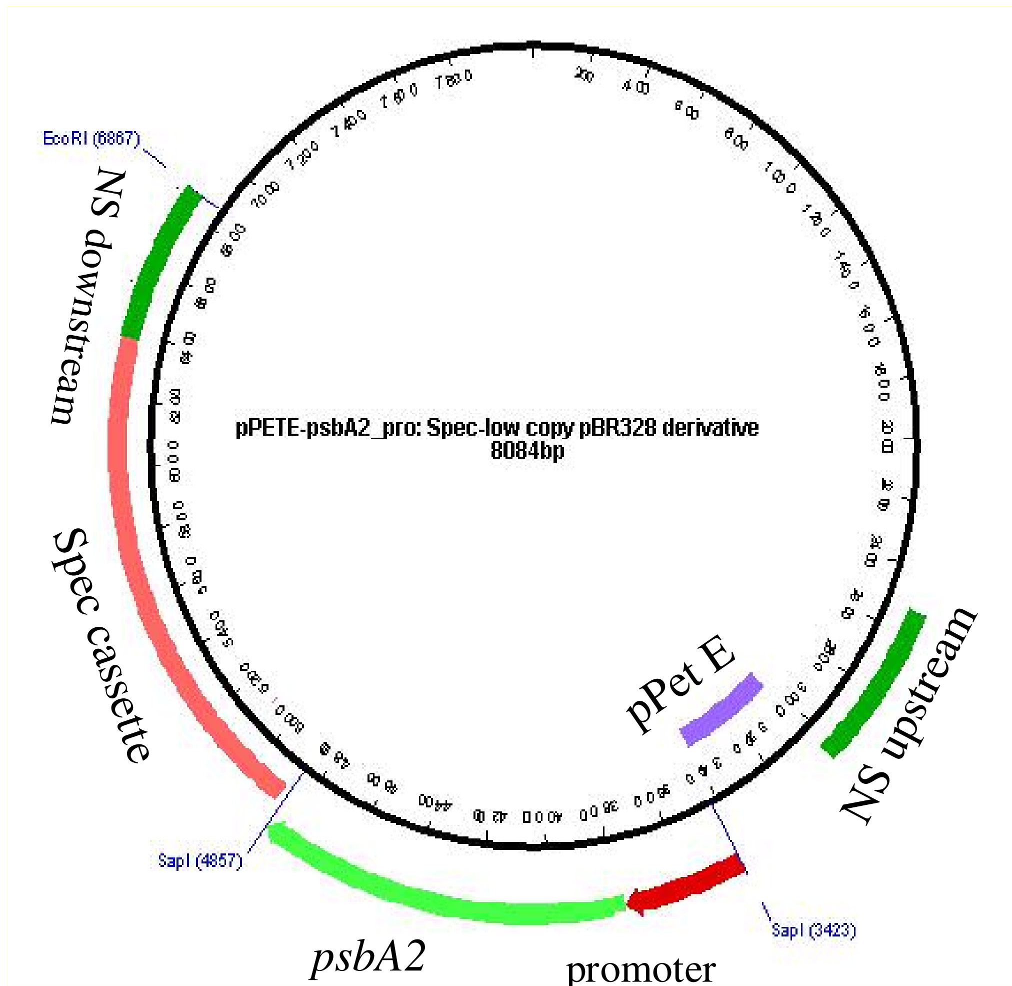


Figure 3.5: Plasmid map of MK2 indicating locations of different fragment positions in the map. For ligation of vector and insert SapI restriction enzyme was used. The vector contains upstream and downstream neutral site regions for transformation of the construct into *Synechocystis* chromosome through homologous recombination. Neutral sites are the regions homologous to the regions of recombination in *Synechocystis* chromosome for introduction of constructs into the host chromosome. pPet E is the copper regulated promoter present upstream to the *psbA2* promoter (300bp), Spec cassette present downstream to the *psbA2* gene with transcription termination region in it. For the confirmation of construct, restriction digestion was used.

The obtained *psbA2* constructs were transformed into *Synechocystis* mutant 4E-3 (in which all three *psbA* genes were deleted) chromosome into the neutral site region (Williams,.1988) (Figure 3.5*)*. This resulted in the prevention of undesirable recombination between the various genomic copies of p*sbA* and introduced *psbA2* gene. The copy of *psbA2* was introduced into the DNA of the host (4E-3) genome by homologous recombination, using a double crossover event as illustrated in Figure 3.4. In this case, selection was on the basis of autotrophic growth since the recipient *Synechocystis* mutant 4E-3 lacks any functional *psbA* genes and therefore requires glucose for growth due to the lack of photosynthesis in the absence of the *psbA* gene. After transformation and selection for autotrophic growth, several colonies appeared and were chosen for further analysis. All colonies appeared to have the same genotype as evidenced by amplification of the NS locus and analyzing the PCR product by restriction analysis. Mutants obtained were named MK1 and MK2. Thus, succeeded in ectopically expressing full length D1 protein under artificial regulation (copper-regulated promoter) for the first time.

Transformation:

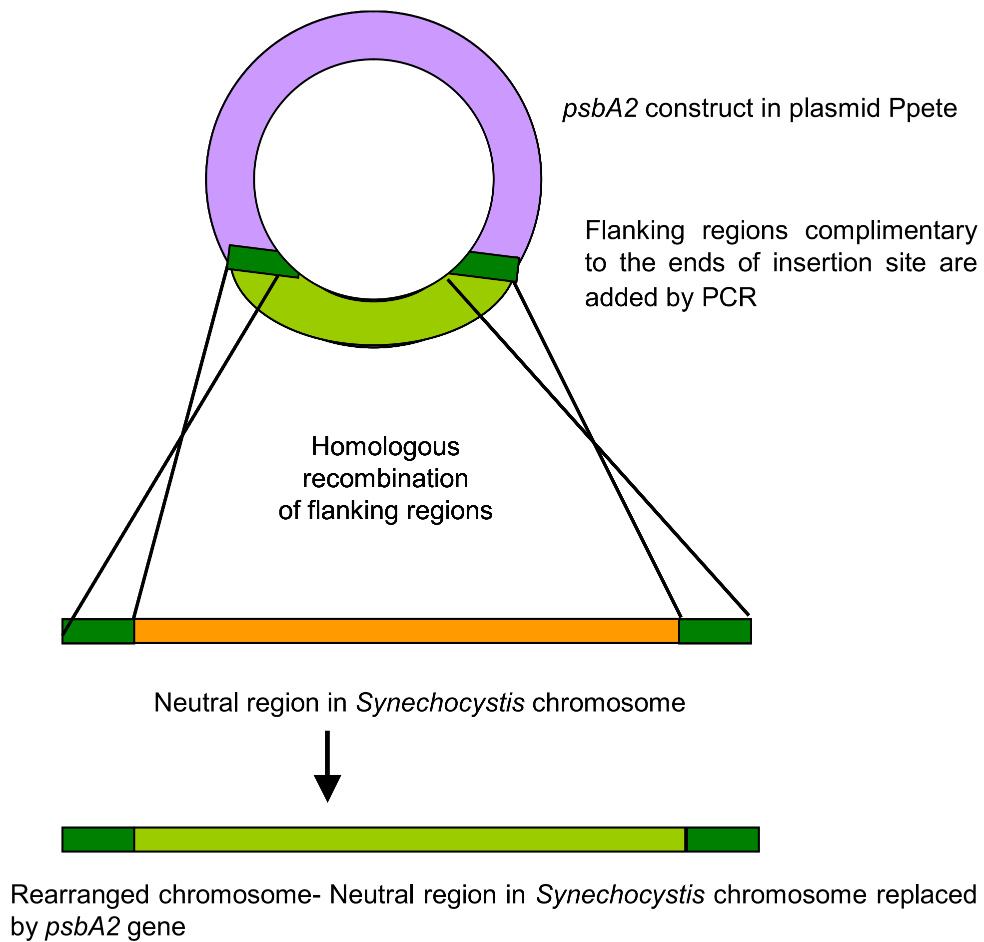


Figure 3.6: Figure representing the process of homologous recombination in *Synechocystis*. Introducing *psbA2* construct into *Synechocystis* neutral site region by homologous recombination using flanking regions (homology regions in construct and in *Synechocystis* chromosome neutral site region).

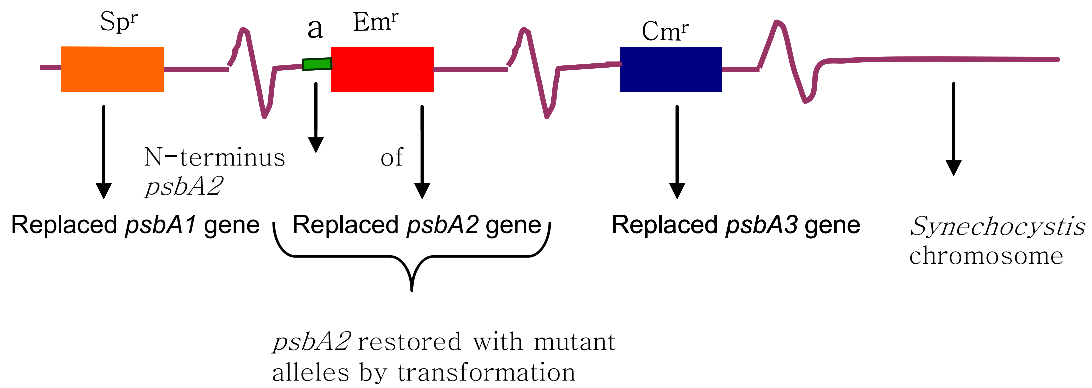


Figure 3.7: 4E-3 strain chromosome structure. 4E-3 is a deletion mutant with all three *psbA* genes replaced by antibiotic resistance genes leaving behind N-terminus of *psbA2* gene, that allow to restore full length *psbA2* gene with transformation of mutant alleles. In *Synechocystis* chromosome *psbA1* gene was replaced by spectinomycin cassette, *psbA2* was replaced by Erythromycin resistant gene and *psbA3* was replaced by chloromphenical resistant gene.

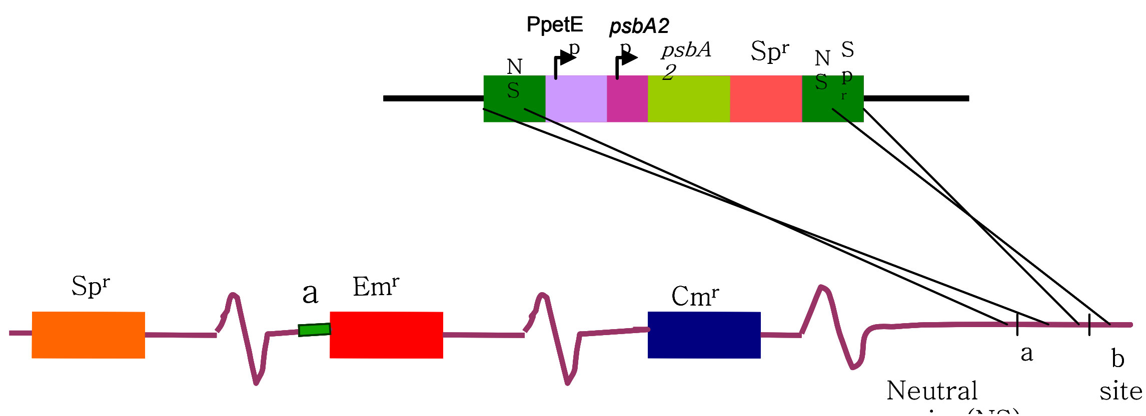


Figure 3.8: The transformation of *psbA2* constructs (Figure 3.3 a & b) with PpetE, *psbA2* promoters and spectinomycin cassette having transcription termination signals by homologous recombination into 4E-3 genome into neutral site region.

Confirmation of transformants:

The introduction of plasmid DNA at expected loci was confirmed by PCR amplification of *psbA2* inserts. For the confirmation of transforments, chromosomal DNA was isolated from strains and was amplified using *psbA2* primers to confirm the size of the fragment.

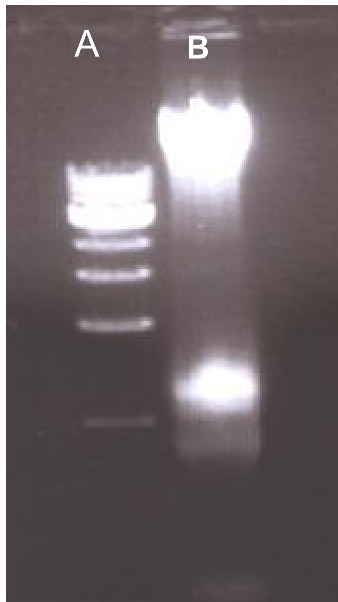


Figure 3.9: Gel picture of the mutant chromosomal DNA, isolated from the transformed *Synechocystis* colonies. Lane A shows marker and Lane B shows the isolated chromosome from the strains.

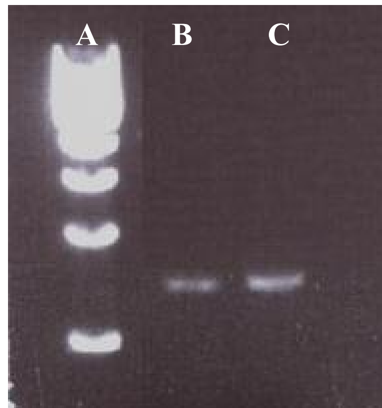


Figure 3.10: The amplified *psbA2* gene from both the mutant strains was loaded onto 0.8% agarose gel to confirm the constructs. Lane A was loaded with marker, Lane B was loaded with amplified *psbA2* product from MK1 chromosome and Lane C was loaded with amplified *psbA2* product from MK2. The amplification resulted in product size of 1038bp. This confirmed my construct.

Amino terminal mutations:

To asses the role of N-terminus and N-terminal cysteine-18 in the turnover of D1 protein N-terminal mutants of MK1 and MK2 were constructed using Stratagene mutagenesis kit as explained in Materials and Methods section chapter II. In the first mutant constructed N-terminal Cysteine-18 was replaced by alanine. PCR base site-directed mutagenesis procedure to introduce aminio acid substitution into the *psbA2* gene. PCR regenerates the original plasmid with a mutation. The mutated plasmid was transformed into *E. coli* for transformation. The plasmid was extracted from the obtained transforments and was digested with the appropriate restriction endonuclease for identification of plasmids containing the mutated sequence. Since silent mutation was introduced into all

the mutagenic primers, successful transforments showed an additional restriction enzyme cut site when compared to that of the control plasmid .This served as a very efficient marker for successful mutagenesis. I confirmed my mutations with sequence analysis.

Characterization of confirmed cysteine mutants using variable fluorescence assay (Figure-2.3) resulted in no significant difference between wild type and cysteine mutant indicating cysteine-18 is not involved in the turnover of D1 protein (Figure 3.11).

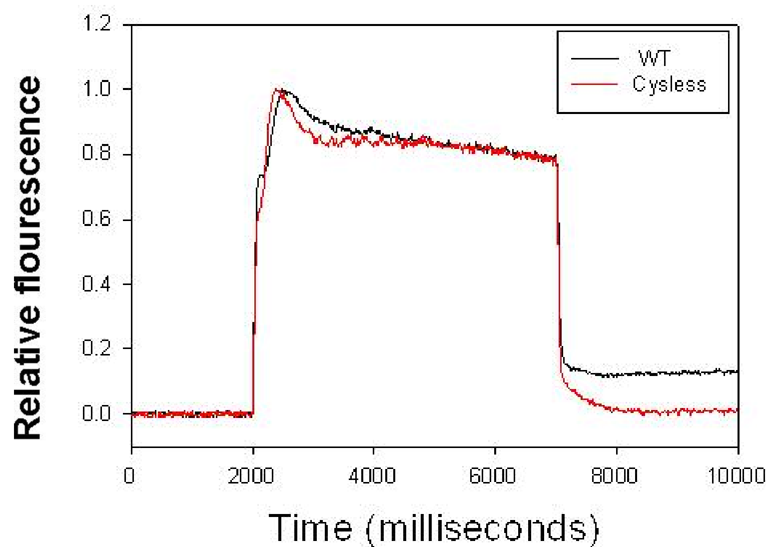


Figure 3.11: Graph representing the variable fluorescence measurement of WT and cysteine less strains. X-axis represents time in milliseconds, Y-axis represents relative fluorescence. The cysteine less mutant is not showing any significant difference in the variable fluorescence than WT, indicating that cysteine-18 is not important in the turnover of D1 protein.

The second mutant constructed was N-terminal deletion mutant in which seven amino acids from backwards were deleted.

While working with N-terminal deletion mutant I was informed that our competing lab was ahead of us in similar project, so I decided not to continue with this.

Characterization:

The strains MK1 and MK2 were characterized by chlorophyll fluorescence studies to identify expression of the D1 protein. MK1 and MK2 resulted in low expression of the D1 protein as deduced from the levels of PSII fluorescence (Materials & methods).

Strain	**Light intensity**	**$Fv = Fm\text{-}F_0/F_0$ (Active PSII concentration)**
MK1	80µmol m^{-2} s^{-1}	0.24
MK1	20µmol m^{-2} s^{-1}	0.57
MK2	80µmol m^{-2} s^{-1}	0.26
MK2	20µmol m^{-2} s^{-1}	0.66
WT	80µmol m^{-2} s^{-1}	0.88
WT	20µmol m^{-2} s^{-1}	0.78

Table 3.1: Variable fluorescence (Fv) of WT and mutant strains MK1 and MK2 at high and low light intensity, suggesting adoption of low light intensity for the growth of mutant strains.

The variable fluorescence Fv measurements provide information about active PS II complexes. The results indicate that MK1 and MK2 strains have low PS II concentration per cell, but posses normal individual PS II composition per cell. The amount of light intensity determines PS II concentration. The mutants are showing low PS II concentration even with WT amino acid sequence. One possible explanation for the observed behaviour of mutants could be the non native location of the *psbA2* gene in mutants resulting in the lower production of steady state *psbA* mRNA, leading to decreased transcription which in turn slows down the synthesis of new D1 protein.

Discussion:

Initially when *psbA2* gene construct (Figure 3.1b) was expressed ectopically under constitutive copper regulated promoter PpetE (palstocyanin) with transcription terminator present in spectinomycin cassette (Figure-3.4) in plasmid pPETE. Copper regulated promoter was thought to increase the expression of protein in the presence of copper in the medium (William J, .200). In our experiments we didn't find any increase in the expression of D1 protein in the presence of copper indicating copper regulated promoter weaker than *psbA* promoter in *Synechocystis.*

When I cloned *psbA2* gene along with 300 base pair *psbA2* promoter under same pPETE plasmid background (Figure-3.5) I do not observed much change in expression. These strains showed lower concentration of PSII per cell. The lower expression even in the presence of *psbA2* promoter may be due to the

reason that length of the promoter region may not be adequate to drive the expression of D1 protein or may be due to the absence of native *psbA2* terminator in the construct. The D1 Cysteine18-Alanine mutant does not represent any significant difference in the PSII concentration when compared to WT indicating cystene-18 is not playing important role in the turnover of D1 protein. While I was working on N-terminal deletion mutant of D1 protein, I was informed that Komenda et al, a competing group of our lab were ahead in N-terminal mutations and we decided not to proceed with the N-terminal mutations.

While characterising the strains MK1 and MK2, they expressed low PSII concentration. Therefore I questioned myself what may be the reason for lower photosystem II concentration in these strains? This diverted the project towards addressing transcript levels for the lower PSII concentration.

Conclusion:

I succeeded for the first time in cloning full-length *psbA2* gene in *E. coli* and, for the first time, ectopically expressed D1 protein under a non-native promoter. While the *petE* promoter that was utilized in the chromosomal construct should, in principal, be regulated by copper, all attempts to obtain conditions resulting in the repression of the gene failed to yield complete shut-off of the expression of *psbA2* at its ectopic location. Therefore, I would recommend that future work ought to be applied towards utilizing an alternative artificial promoter now that I have shown that ectopic expression works.

An important finding, and one that lead to the experiments described in the next chapter, was that both of the ectopic psbA2 expression strains had PSII activity that was low and very light sensitive in *Synechocystis*. Even when I used the *psbA2* promoter (300 base pairs) (MK2) in the same vector pPETE, the strain failed to show much change in photosystem II wild-type activity. The basis for these observations was explored by studying the damage and repair properties of the strains as shown later. Finally, I found that another group was ahead in the construction and analysis of D1 N-terminal mutations. Therefore, I decided not to proceed with this aspect of the project and instead focused upon a physiological characterization of the ectopic *psbA2* strains I had already constructed. As described below, the observed light-sensitivity and lower PSII concentrations in the strains MK1 and MK2 in conjunction with the idea that the *petE* promoter is not likely as active as the native *psbA2* promoter led us to think about a possible correlation between *psbA2* transcript levels and the capacity to repair PSII following photoinhibition.

CHAPTER-IV

Studies of damage and repair of D1 protein

Introduction

The objective of my research was to construct a mutagenic system that allows for the mutation of entire D1 protein including the N-terminus of the D1 protein. My research findings after characterizing the mutant strains with fluorescence studies indicated the concentration of photosystem II (PSII) in mutant strains was lower than WT. Therefore, I was further interested in assessing the factors that limit the rate of repair of PSII and to test my hypothesis that 'transcript levels restricts the rate of repair.

To address this hypothesis, I conducted fluorescence assays in conjunction with real time PCR. For this study, four different strains were used, WT and WT* were used as control strains. WT* is a deletion mutant having only *psbA2* gene (WT *psbA2* sequence) in *psbA2* native location in the host chromosome**.** MK1 and MK2 are mutant strains (Figure 3.3, 3.4, 3.5). In this chapter, I will describe and discuss the results of my experiments in testing my hypothesis.

Results and discussion

In this study, four different strains were subjected to high light treatment (photoinhibition) for 60 minute, and then subsequently shift to growth light for recovery to investigate the rate of recovery in these strains. By this experimental design, the damage and recovery rates of MK1 and MK2 were compared with that of WT and WT* as controls. To investigate the damage and recovery rate, chlorophyll fluorescence measurements were used. The measurement provided information about active PSII complexes and active charge separated centers (intact D1 proteins) during photoinhibition and recovery. In preliminary experiments, when the photoinhibited WT cells transfer to low light condition for recovery, I observed that the rate of recovery is inverse proportional to the extent of photoinhibition. I adopted 1000 µmol m^{-2} s^{-1} light intensity as photoinhibotory condition. When the cells were exposed to photoinhibitory condition for 30 minutes or 60 minutes, the rates of recovery was 95% or 80 % in 120 minutes, respectively. However, when the exposure of high light was for 90 minutes, the cells did not survive. Therefore, 60 minutes was suitable for ideal time for photoinhibition to determine the efficiency of repair. The cells were not irreversibly damaged, so that I can observe the rate of recovery.

When mutants were exposed to 1000 µmol m^{-1} s^{-1} light intensity, the cells did not survive. Chapter-III, Table 3.1 shows the light sensitivities of MK1 and MK2 indicating that the mutants need low light intensity for the accumulation of the D1 protein (recovery) as evidenced by PSII fluorescence. When I adopted the

800 μmol m^{-1} s^{-1} light intensity of MK1 and MK2, extents of damage in MK1 and MK2 cells were similar to those of WT and WT*. The results support that lower levels of PSII exhibit more sensitivity to high light and undergoes damage even under low light intensity in *Synechocystis*. Presumably, PSII acts as a sink for light excitation and once the population of PSII is fully damaged, then further light excitation is mainly dissipated by yielding products that are lethal for cells.

Two types of fluorescence assays were used as explained in chapter II, Materials and Methods to estimate the activity of cells. F_v represents activity of functional PSII to measure electron transfer rate from water and plastoquinone, as the endogenous electron donor and acceptor, respectively. Thus, F_v can be considered to be proportional to the overall functioning of the entire PSII complex. On the other hand, $F_{HA\text{-}QA}$ means charge separated activity of PSII reaction centers to measure electron transfer rate from artificial electron donor, hydroxyl amine (HA), to Q_A. To measure the $F_{HA\text{-}QA}$, HA, as artificial electron donor, was added to substitute water and electron transport inhibitor was added to inhibit electron transport beyond the first stable electron acceptor, Q_A. Therefore, the $F_{HA\text{-}QA}$ assays only the charge-separating activity of PSII reaction center. $F_{HA\text{-}QA}$ should include both functional and damaged PSII centers. When I used chemicals for measuring $F_{HA\text{-}QA}$, $F_{HA\text{-}QA}$ provided the very similar fluorescence pattern of information for recovery and damage in all strains as F_v (Fig. 4.1). The yield of damage was approximately 10% less and the recovery rate was 10% more in all strains at same time point in $F_{HA\text{-}QA}$ (active D1) in comparison to Fv (active PSII). In the case of F_v, it provides information about the

active PSII complexes. During recovery the formation of active PSII complexes is a rate limiting step, as all the cofactors and coenzymes have to assemble to form the final active PSII complexes (Figure 1.1). During damage, F_v will go down fast compared to $F_{HA\text{-}QA}$, as the PSII complexes will disassemble first at high light intensity, still there is charge separation providing high $F_{HA\text{-}QA}$. This explains that the damage of PSII is a result of damage to D1 protein. I used F_v data for more data analysis. Because, changes of the $F_{HA\text{-}QA}$ and F_v were very similar during both photoinhibition and recovery in all strains after normalization (Figure 4.3).

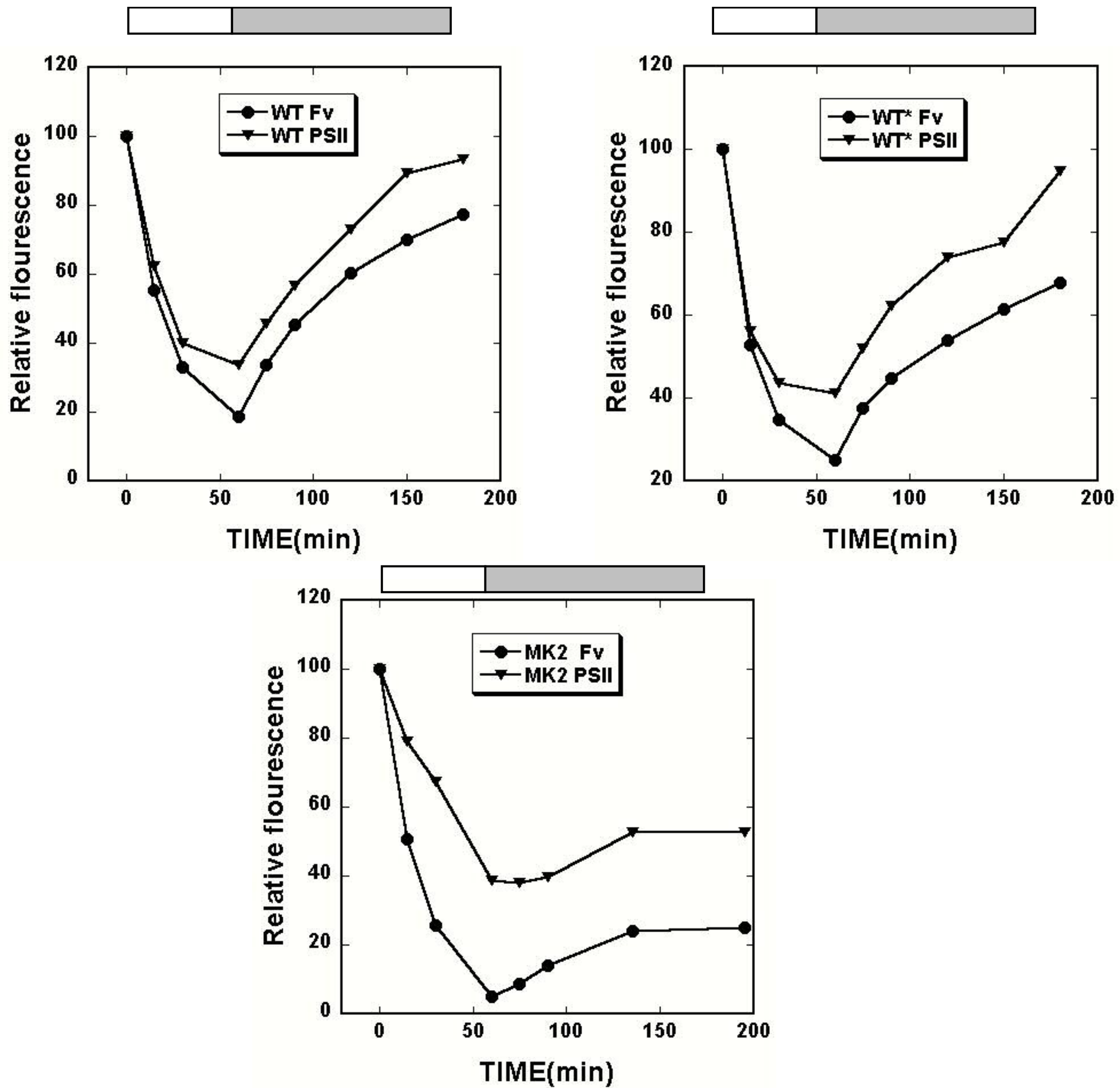


Figure 4.1: Changes of Fv and $F_{HA\text{-}QA}$ during photoinhibition and recovery. The damage rate (represented by white box) was 10% less and the recovery rate (represented by black box) was 10% more at same time point in $F_{HA\text{-}QA}$ (active D1) in comparison to F_v (active PSII). This indicates the damage to photosystem II is a result of damage to D1 protein of photosystem II. Three biological replicates and two technical replicates were used for data collection. The error bars are small and are masked by marker point.

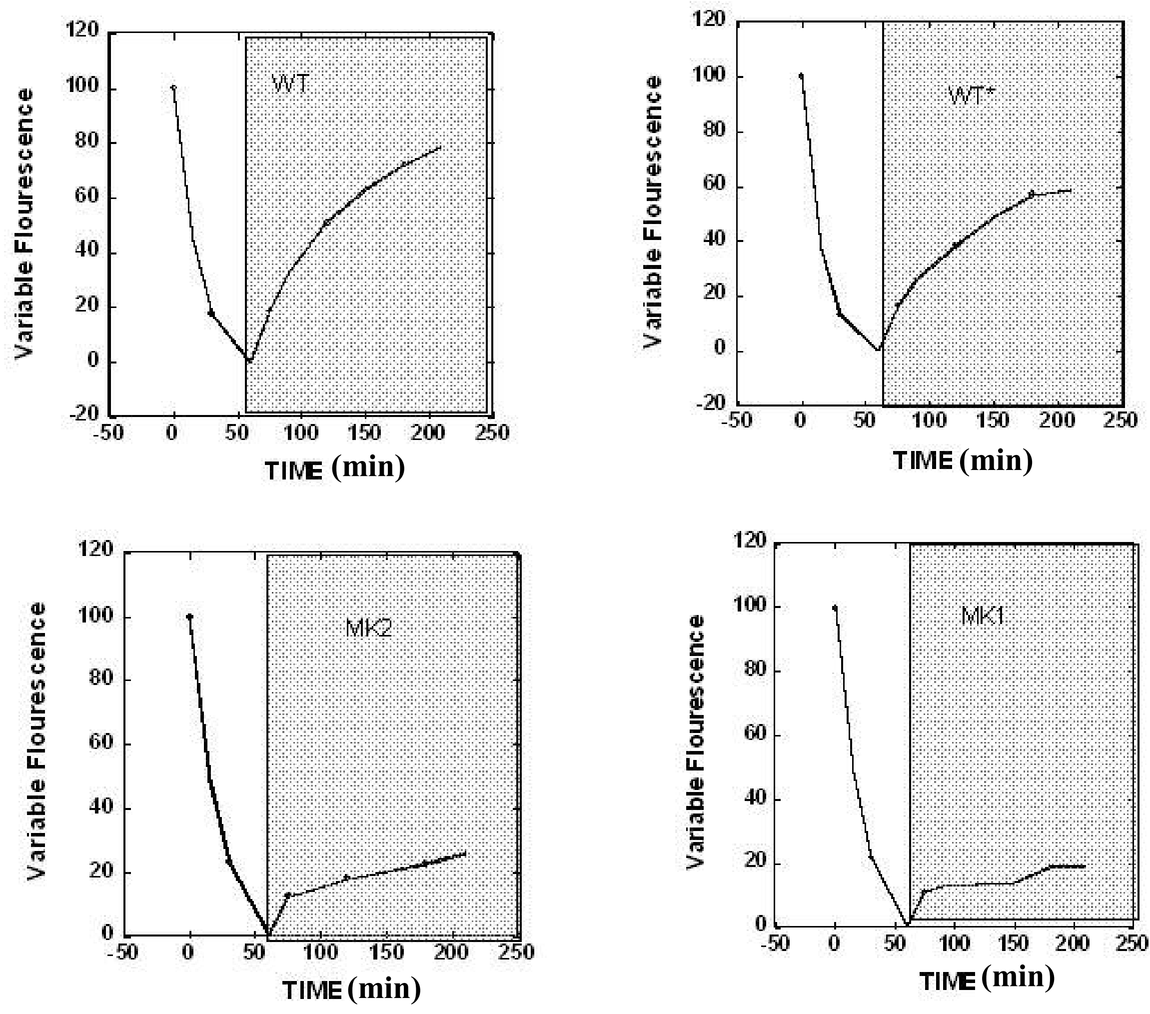


Figure 4.2: Changes of normalized Fv during photoinhibition and recovery. When the cells were photoinhibited and transferred to low light (growth light) (white box) to observe their recovery (black box), 80% PSII recovery in WT, 58% recovery in WT*, 22% recovery in MK2 and 18% recovery in MK1 was observed. Three biological replicates and two technical replicates were used for data collection. The error bars are small and are masked by marker point.

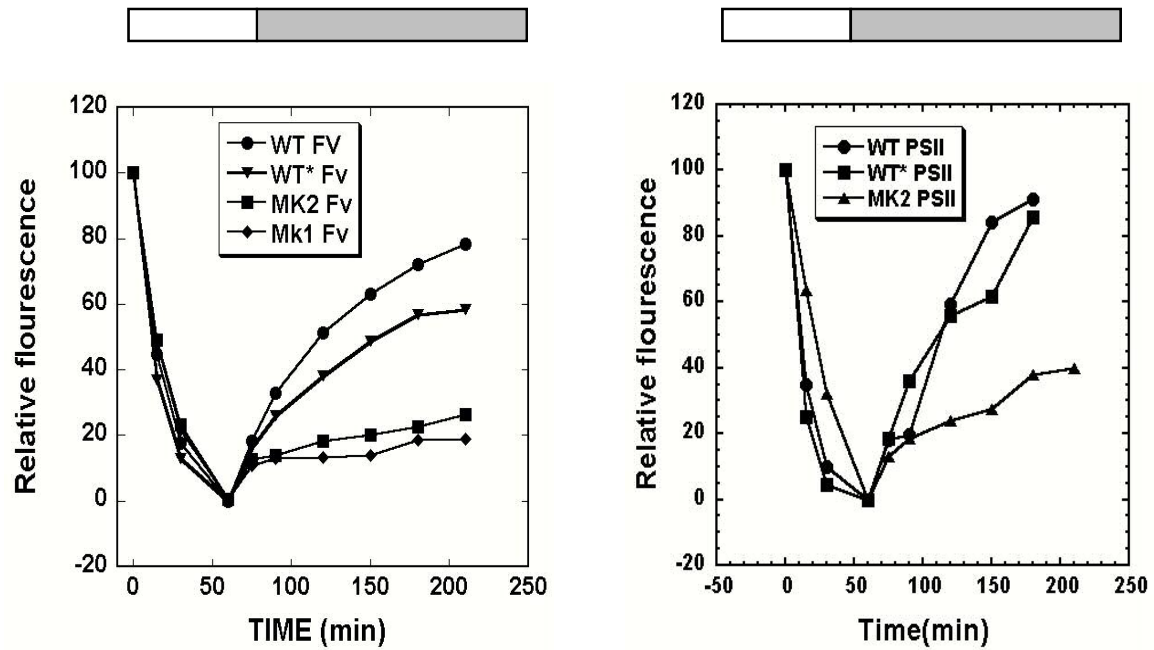


Figure 4.3: Comparison of photoinhibition(white box) and recovery (black box) results of all strains, indicating that the Fv and $_{FHA\text{-}QA}$ provide same pattern of information. From the fluorescence assays the recovery rates were WT > WT* > MK2 > MK1. Three biological replicates and two technical replicates were used for data collection. The error bars are small and are masked by the points.

From F_v results of Figure 4.3, damage rate constant (K_D) and recovery rate constant (K_R) of all strains was calculated. Half life of damage and recovery was calculated by using the following formula

$$t_{1/2} = 0.693 / (K_D) \text{ or } 0.693 / (K_R)$$

Damage Rate:

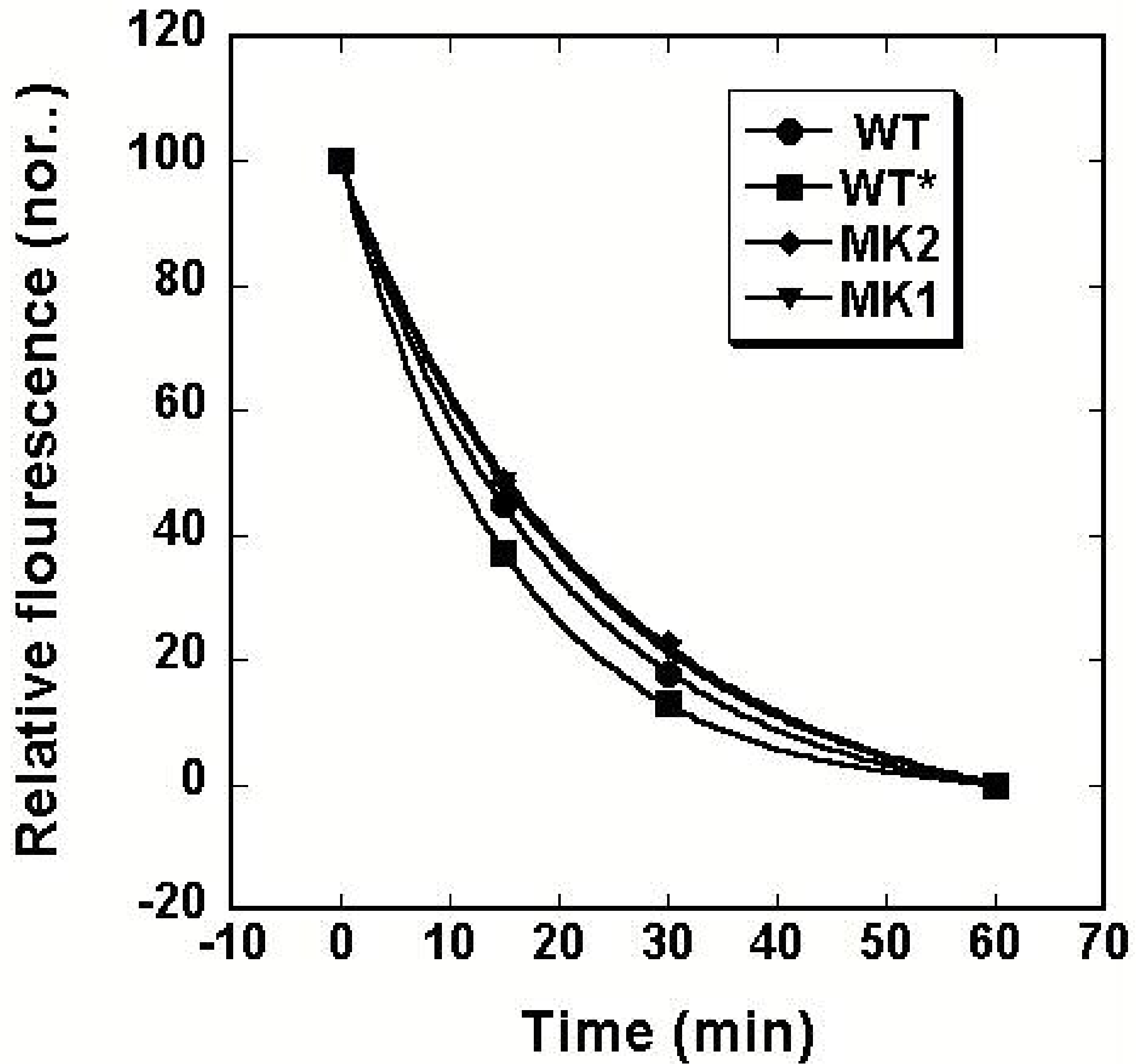


Figure 4.4: Differences of Fv during photoinhibition in WT, WT*, MK1, and MK2. In the figure, there is no significant difference in damage rate among strains. . All data were normalized. Three biological replicates and two technical replicates were used for data collection. The error bars are small and are masked by the points.

Strain	Damage constant (K_D)	Half Time (minute)	Relative Half Time (%)
WT	0.049994	14.14	1.0
WT*	0.063794	11.00	1.28
MK2	0.041834	16.90	0.83
MK1	0.04341	16.11	0.87

Table 4.1: Comparison of damage rate constant (K_D) of all strains. K_D was obtained from curve fitted data of Fv graph during photoinhibition (Fig. 4.4).

In photoinhibition and recovery experiments, I wanted to photoinhibit all the strains to the same extent to compare the rate of recovery. My experimental results indicate that the strains do not represent significant difference in the rate of damage (Table 4.1, Figure 4.4), indicating the same photoinhibitory treatment to all strains. This allowed the comparison of the rates of recovery of these strains to one another.

Rate of damage measurement with lincomycin (protein synthesis inhibitor):

Lincomycin is a protein synthesis inhibitor. When I added lincomycin to the culture, it inhibited the D1 protein synthesis, therefore I was able to measure the rate of damage alone. All experimental procedures are same as the previous experiment, except add lincomycin. The data indicates the damage of MK2 is faster than the damage of WT at the same light intensity (Figure 4.5). This indicates the light sensitivity of the strains (Table 3.1) due to low PSII concentration. Significant differences of damage rate between WT and MK2 were not observed (Table 4.2). The result support that damage rate was very faster than the repair rate.

Strain	Damage constant (K_D)	Half time (minute)	Relative half time (%)
WT-lin	0.052	13.00	100
WT+lin	0.042	16.50	126
MK2-lin	0.050	13.86	106
MK2+lin	0.074	9.36	72

Table 4.2: Represents the relative half time of damage alone, when there is no repair. The damage constant was obtained by curve fitting of photoinhibition relative fluorescence time points. The rate of damage of WT and MK2 are not significantly different.

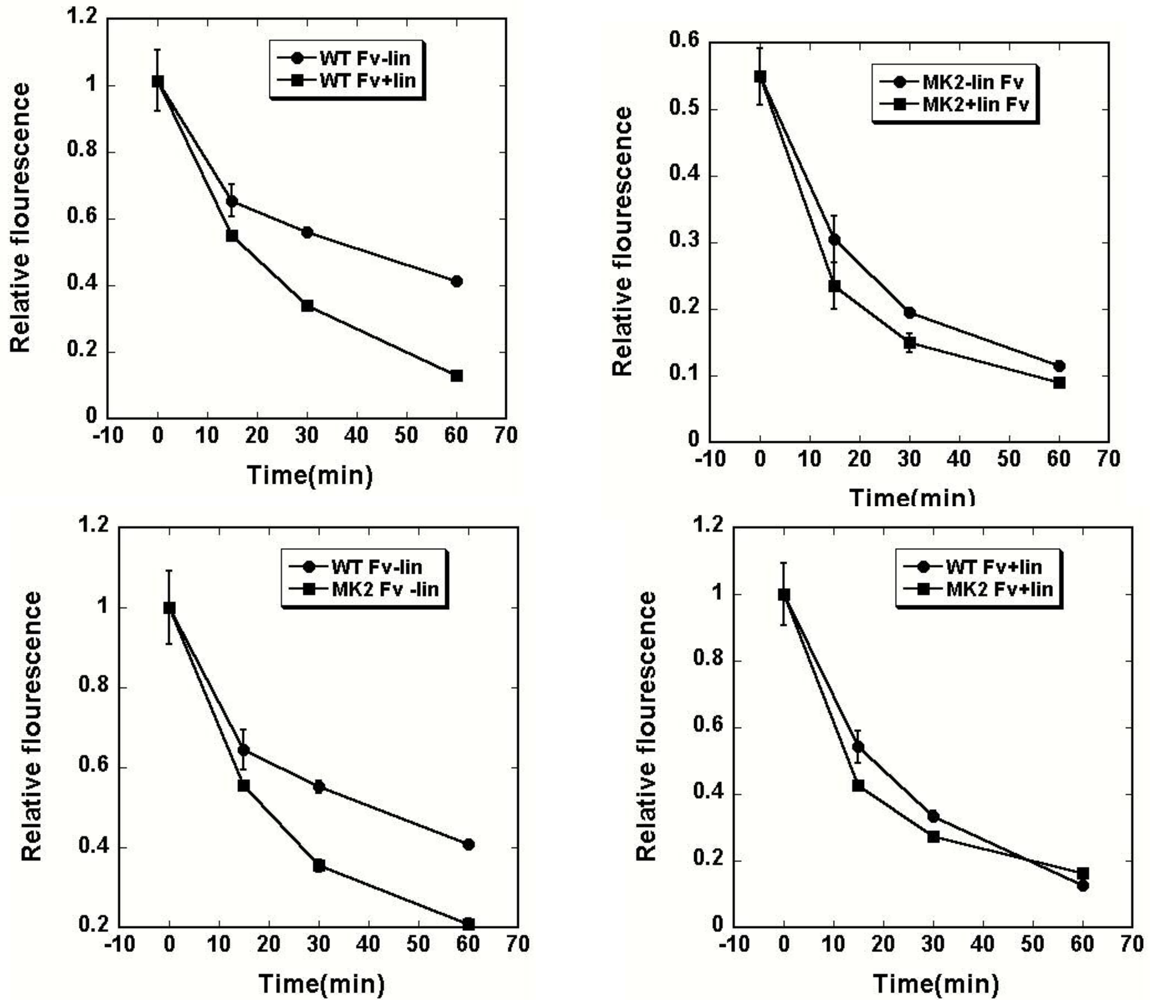


Figure 4.5: Graphs representing the damage of strains. The data is collected with the addition of lincomycin to the test sample. Lincomycin is a protein synthesis inhibitor, that allows to measure the rate of damage alone, with inhibition of protein synthesis. The damage rate of MK2 was more than WT without lincomycin indicating the lower repair rate. The rate of MK2 was more than Wt even with lincomycin in the culture, indicating the light sensitivity of strains due to low PSII concemtration and low D1 production.

Recovery rate:

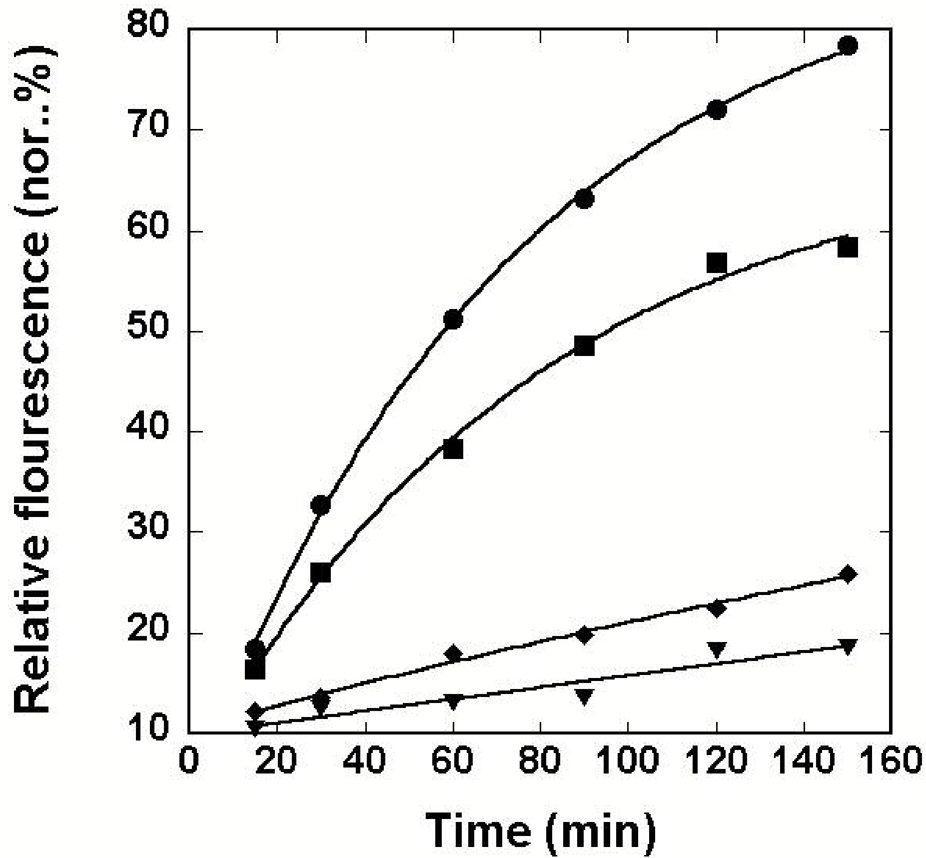


Figure 4.6: The rates of recovery rates of PSII in control strains WT, WT* and mutant strains MK1 and MK2 are shown in the figure. The figure illustrates WT* recovery rate approximately similar to WT and MK1 recovery rate approximately similar to MK2 and the recovery rates of mutants are significantly lower than the recovery rates of control strains. The recovery rates are obtained from the variable fluorescence measurement of photoinhibitory experiment after transfer to low light intensity of 20 µmol m^{-1} s^{-1} for recovery. Fluorescence assay for variable fluorescence measurements was performed as described in Materials and Methods. The difference between minimum and maximum fluorescence was normalized to 100% as shown in Table 4.2 in all strains using the formula ((X value-minimum value) / (maximum value-minimum value)).

Strain	Recovery constant (K_R)	Half time (minute)	Relative half life (%)
WT	0.013	53.30	1.0
WT*	0.012	57.75	0.92
MK2	0.001	693	0.77
MK1	0.0003	2310	0.2

Table 4.3: Recovery constant (K_R) of strains obtained from curve fitting of recovery time points is used to calculate the relative half time of strains.

The result of figure 4.6 indicates that the rate of repair of MK1 and MK2 is lower than that of WT and WT*. After photoinhibition, WT and WT* recovered 80-85% of activity of the PSII before photoinhibition after 3 hours recovery. But, MK1 and MK2 only restored 20-22% of the PSII activity. The recovery in the control strains beyond 4 hours was not noticeable as they reached maximum fluorescence and stayed steadily.

The rate of recovery of PSII activity previously was shown to reflect rate of D1 synthesis and replacement in damaged PSII (Figure 1.4). Therefore, I further examined that the reason for slow recovery is caused by retard recovery of impaired D1 in mutants by observing the levels of mRNA in controls and mutants.

For determining mRNA levels quantitative real time PCR was used. Amplification was detected by the use of fluorescent dye SYBR Green. *rnpB* gene was selected as internal standard for my experiment. The *rnpB* gene expresses the same copy number under any experimental conditions.

A Standard curve was constructed by using WT cDNA 10 fold dilutions (Figure 2.5) using both *psbA2* gene and standard gene *rnpB*. The ideal concentration for the experiment was determined to be 50 ng. The same concentration was adopted for other samples of cDNA, as *psbA2* gene is highly expressed in all samples. A standard curve was constructed by plotting concentration of cDNA against Ct values (Figure 4.7 and 4.8). Ct is the cycle number at which the log (cycle) set threshold is reached. PCR efficiency was 90% and linear correlation was 0.998. *psbA2* and *rnpB* primers resulted in a single narrow peak indicating pure product (Figure 4.10).

The normalization of the Ct value for target gene with the Ct values of the reference gene is described in “real time data processing” chapter-II.

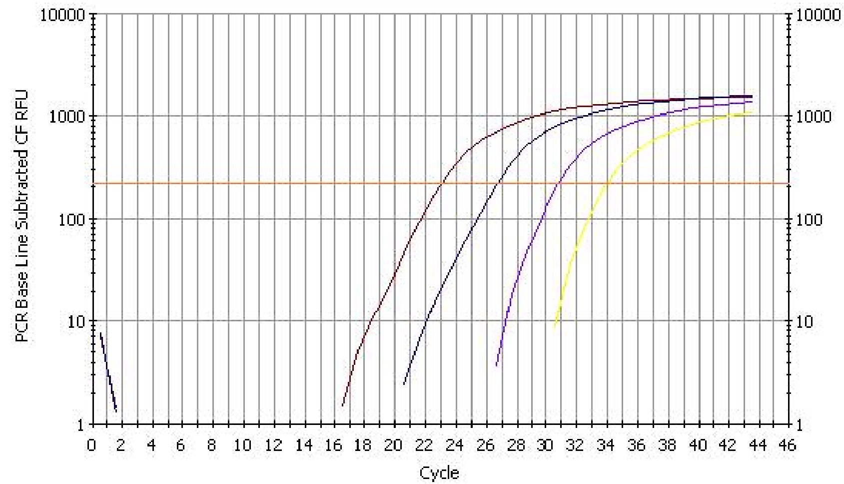


Figure 4.7: Graph representing the amplification curve of *psbA2* gene constructed by using WT cDNA. The amplification graph is a result of plotting PCR cycle number on X-axis against fluorescence intensity on Y-axis. The orange color line shows the set threshold for calculation of Ct values. The colored raising curves represent the amplification cycles of 10-fold dilutions of WT cDNA using *psbA2* gene starting from 100ng/µl. More dilute sample will amplify later than the concentrated sample. The extra line at the starting of graph represents the background noise.

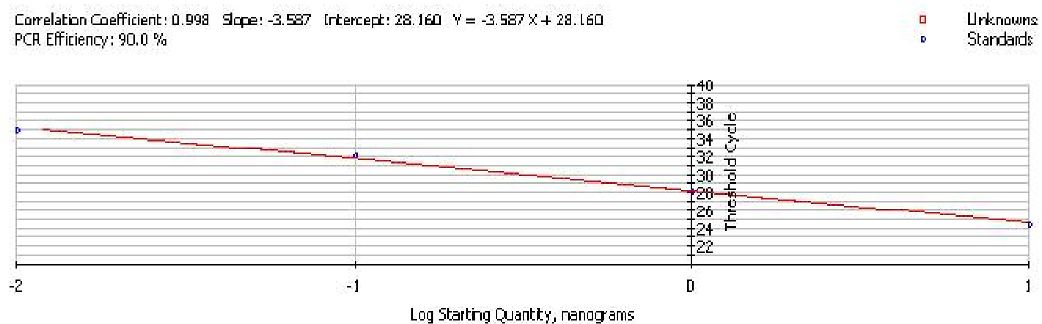
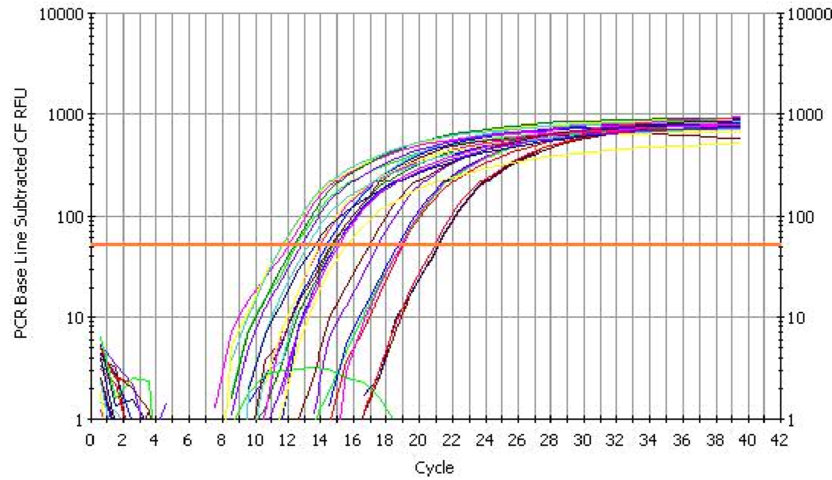


Figure 4.8: Graph representing the standard curve showing the log of the concentration of cDNA vs. threshold cycle for *psbA2* primer. The graph is linear indicating that the threshold cycle number is proportional to the amount of cDNA and the slope of the graph represents PCR efficiency.

a) Amplification curve for *psbA2* primer.



b) Amplification curve for *rnpB* primer

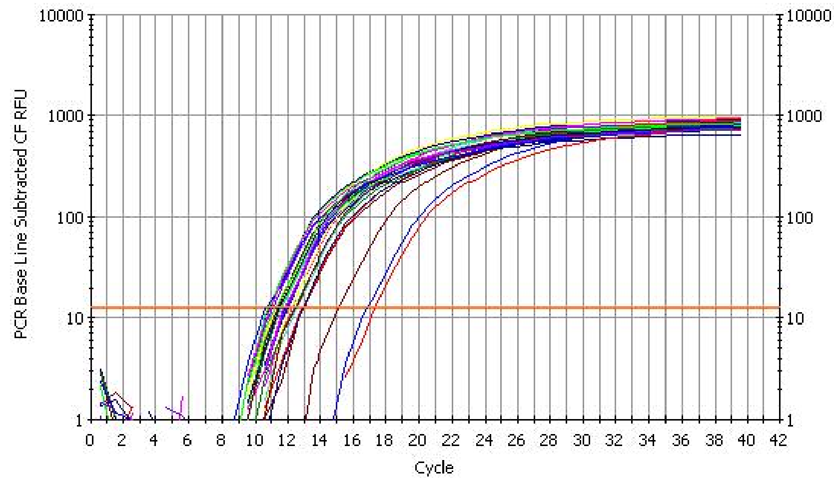
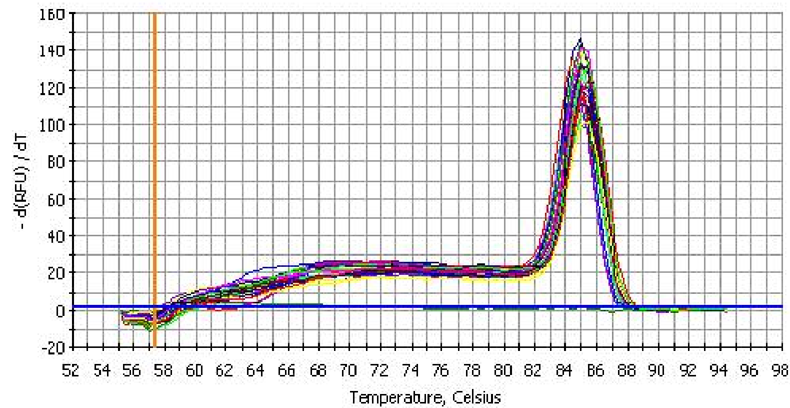


Figure 4.9: The amplification cycles of a) *psbA2* primer and b) *rnpB* primer. The amplification graph is a result of plotting PCR cycle number on X-axis against fluorescence intensity on Y-axis. Orange line represents the set threshold for calculation of Ct values. The cDNA copies are used in triplicates for the reaction in a 25 µl reaction. The curves represent the amplification graphs from a group of wells, in which same primer pair was used. Small irregular lines near the starting point of graph represents the back ground noise.

a) Melt Curve Graph for *psbA2* primer



b) Melt Curve Graph for *rnpB* primer.

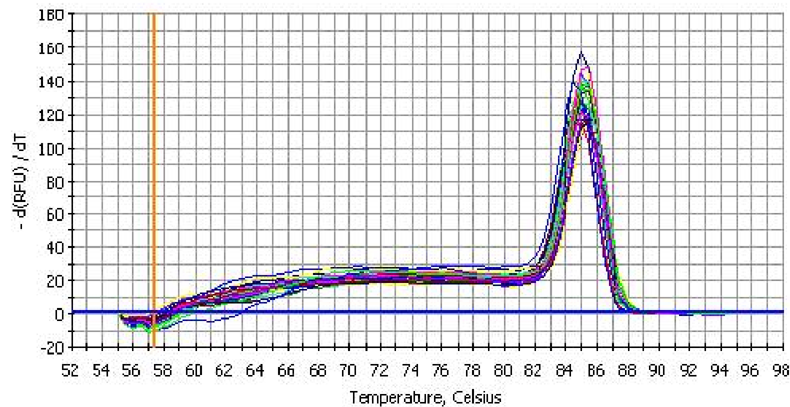


Figure 4.10: The melt curve graphs for a) *psbA2* primer b) *rnpB* primer representing single narrow peak. Melt curve is obtained as a result of plotting temperature against fluorescence intensity. Melt curve is a result of melting or separating of the two strands of the target dsDNA at a particular temperature resulting in the release of SYBR Green. Each DNA has its own melting temperatures based on the number of base pairs and GC content, if the melt curve is a result of the target DNA, it results in a single narrow peak. Multiple peaks represent contamination of the product. This data represents set of melt curves of the target DNA when I used in different wells, indicating purity of the product.

I subjected the cDNA of all four strains to real time PCR experiment to investigate the effect of *psbA2* transcript levels on the rate of recovery. In this study transcript levels of four different strains (control strains and mutant strains) were compared. Representative results of real time PCR indicated lower level of transcripts in MK1 and MK2 than that of WT and WT* before photoinhibition. An increase in transcript levels was observed after recovery in WT and WT*, while transcript levels of MK1 and MK2 decreased at same time (Figure 4.11). As shown in Figure 4.12, the recovery half time has strong correlationship with transcript levels.

Transcript levels normalized to wild-type *psbA* mRNA before photoinhibition (Steady state) and 60 minutes after photoinhibition (60 minute recovery).

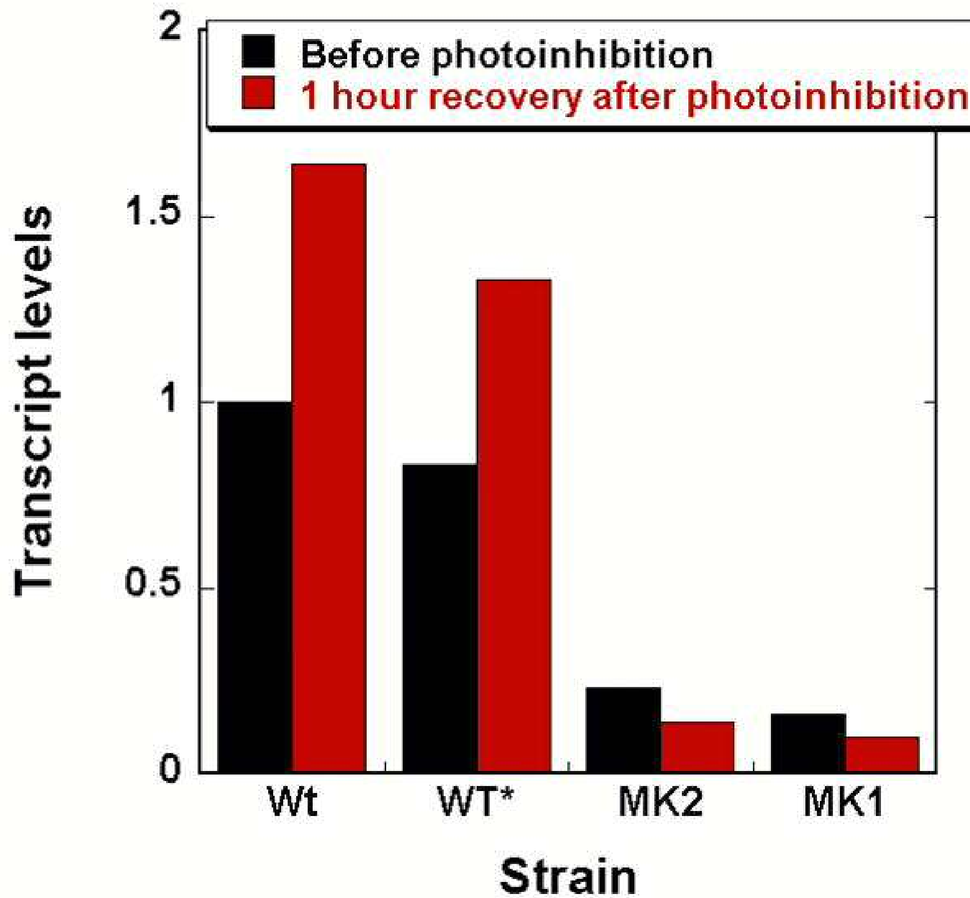


Figure 4.11: Graph representing the transcript levels before photoinhibition and one hour after photoinhibition. X-axis represents the names of strains, Y-axis represents the transcript levels. The transcript levels of control strains WT and WT* are approximately parallel to each other and mutant strains MK2 and MK1 are approximately parallel to each other. The transcript levels of mutant strains are lower than the transcript levels of control strains. Sixty minutes after photoinhibition (recovery) the transcript levels of control strains WT and WT* increased while the transcript levels of MK2 and MK1 decreased.

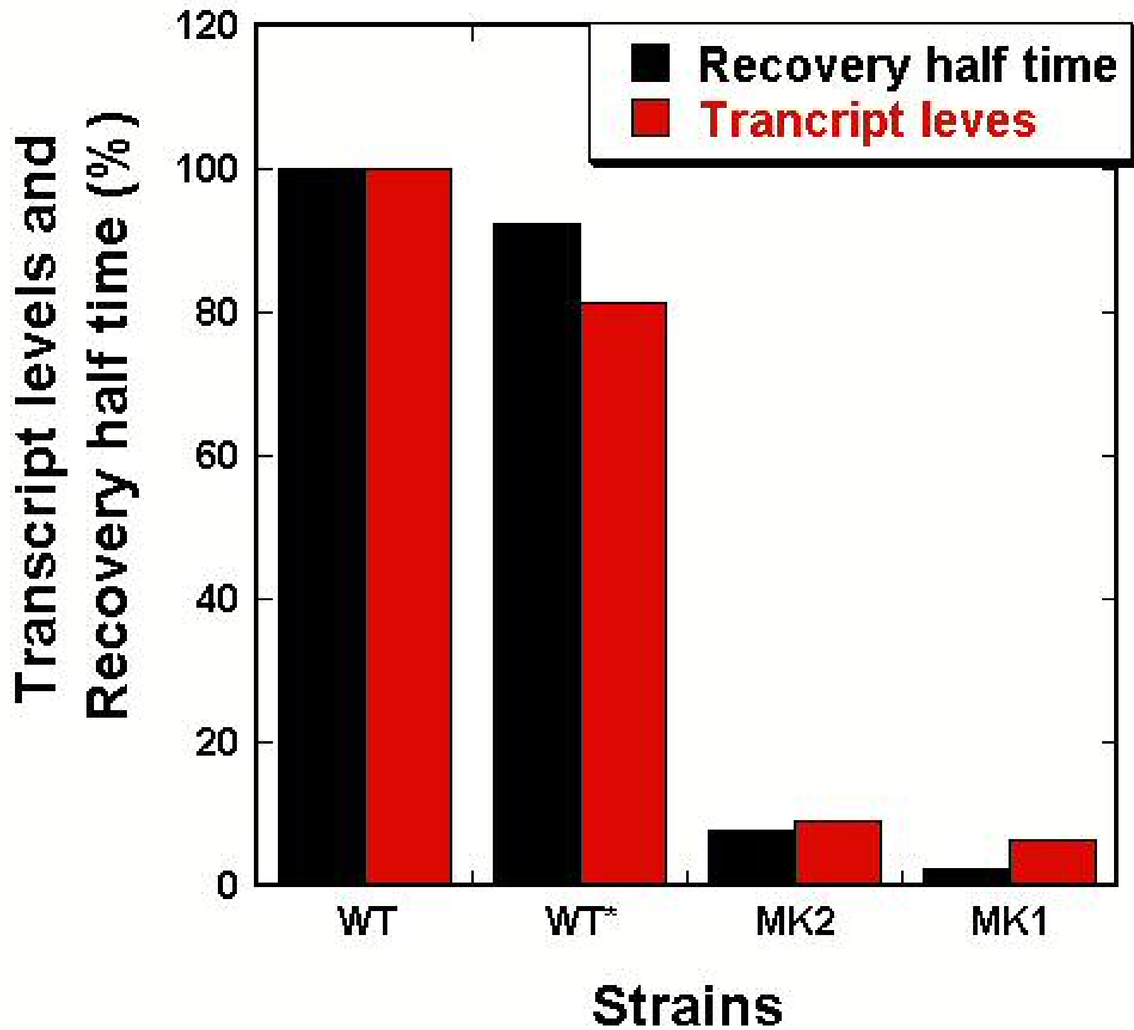


Figure 4.12: Graph representing the transcript levels and t $_{1/2}$ of recovery in control strains WT, WT*, MK1 and MK2. The rate of recovery in different strains is directly proportional to level of transcript (normalized values). The mRNA levels of strains are obtained from the analysis of real time PCR data as described in Materials and Methods. The recovery rate constant was used to calculate half life of damage and as shown in Table 4.2. Recovery rate constant (K_R) obtained from the curve fitting slope of photoinhibitory variable fluorescence time points obtained from fluorescence assay. The fluorescence assay for photoinhibition fluorescence measurements was performed as described in Materials and Methods. The mRNA levels and variable fluorescence rates of WT*, MK2 and MK1 are normalized to the values of WT considering WT rate as 100%.

Gene dosage effect is that the number of copies of gene that change their effect based on the level of expression. Analysis of the gene dosage effect in different conditions revealed the gene expression levels change according to gene levels (Hu et al., 2005; Li et al., 2002). *psbA2* gene is highly expressed gene as a result of very strong *psbA2* promoter in chloroplast genome and cyanobacterial genome (Williams,.1988). In present study, *psbA2* gene dosage limits the levels of *psbA2* transcript leading to decreased production of D1 protein. The decreased production of D1 protein is responsible for decrease in the recovery rate of mutants MK1 and MK2 in comparison to control strains WT and WT*. The research of Komenda et al. (2000) on correlation between transcript levels of *psbA3* and D1 protein synthesis in *Synechocystis* are in agreement with the statement that the transcript level regulates the rate of repair. In my study, I wanted to pursue this more extensively by studying the transcript levels of *psbA2,* highly expressed gene in *Synechocystis* in comparison to recovery rate (Figure 4.12).

Fluorescence assays in photoinhibition and recovery showed that the rate of repair is slow in MK1 and MK2 in comparisons to WT and WT*(Figure 4.2). The real time PCR data showed low *psbA2* transcript levels in MK1 and MK2 before photoinhibition and during recovery in comparison to WT and WT*. It seems more likely that the retard repair rate of MK1 and MK2 results from lower levels of transcript of MK2 and MK1.

In WT and WT*, the transcript levels after 60 minute recovery are up regulated. The transcript levels of MK1 and MK2 are significantly low, compared

to WT and WT* (Fig. 4.11). Overall the transcript levels followed the same trend as recovery rates of PSII; in other words the transcript levels are closely proportional to the rate of repair (Fig. 4.12).

This indicates that *psbA* driven by full-length native promoters (Control strains-WT and WT*) are increased but non-native promoters (copper regulated promoter-MK1) and partial psbA2 promoter (300bp *psbA2* promoter-MK2) are not. Independent experiments in the lab showed increase in transcript levels of control strain WT reaches maximum within 15-30 min after high light exposure. The rate of recovery in different strains is closely proportional to level of transcript. It seems more likely that the observed lower repair rate is a result of lower levels of transcript in MK2 and MK1 (Figure 4.12).

The lower transcript level in MK2 and MK1 can be due to the position of *psbA2* gene in its non native chromosomal location and there is a potential for copper regulated promoter to be weaker than *psbA* promoter in *Synechocystis*. When I used *psbA2* promoter (-300 base pairs), the length of the promoter may not be adequate to drive the expression of D1 protein and the possibility of the absence of native terminator resulted in decreased *psbA2* levels and light sensitivity of strains.

Lower levels of transcript support the hypothesis that the *psbA* mRNA levels limit the rate of PSII repair and this explains the light sensitivity of these strains. The lower levels of transcripts leads to decrease in new D1 protein synthesis leading to decreased rate of repair of damaged PSII complexes.

An essential task for the repair of PSII is degradation of damaged D1 protein, which is correlated with the synthesis of new D1 protein. The damaged PSII complexes needs to be repaired before the PSII lost full function, to avoid more damage. The newly synthesized D1 protein needs an empty slot for insertion into damaged PSII. This needs the degradation of damaged D1 protein for making empty slot in PS II. Therefore the rate of degradation is coupled to the rate of synthesis of new D1 protein (Komenda et al., 2000). In MK1 and Mk2 strains lower *psbA2* transcript levels are providing low mRNA levels leading to decreased rate of translation since there are fewer messages for ribosomes to read. This results in a reduction of D1 protein synthesis causing the accumulation of damaged D1 protein within the PSII complex (Figure 4.13) as the rate of damage is more than the rate of D1 protein synthesis, leading to low repair rate.

Proposed Model for Difference in WT and MK2 Recovery rates

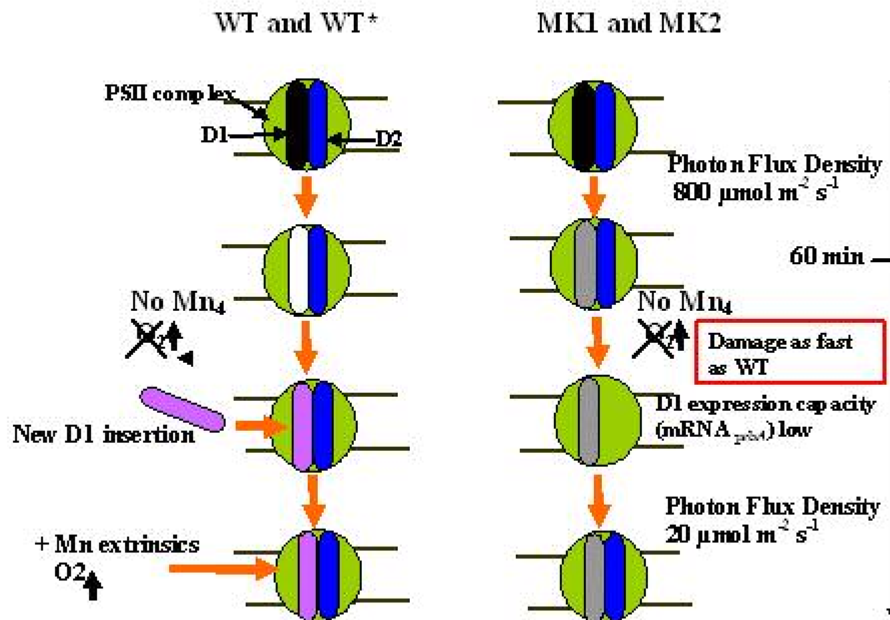


Figure 4.13: Figure illustrates the Proposed Model for Difference in WT, WT*, MK1 and MK2 Recovery rates. The rate of damage is very similar in all strains at 60 minute photoinhibition. The reason for different rates of recovery may be due to low D1 expression capacity in MK1 and MK2 strains when compared to WT and Wt* as a result of low mRNA levels.

Flow chart of discussion

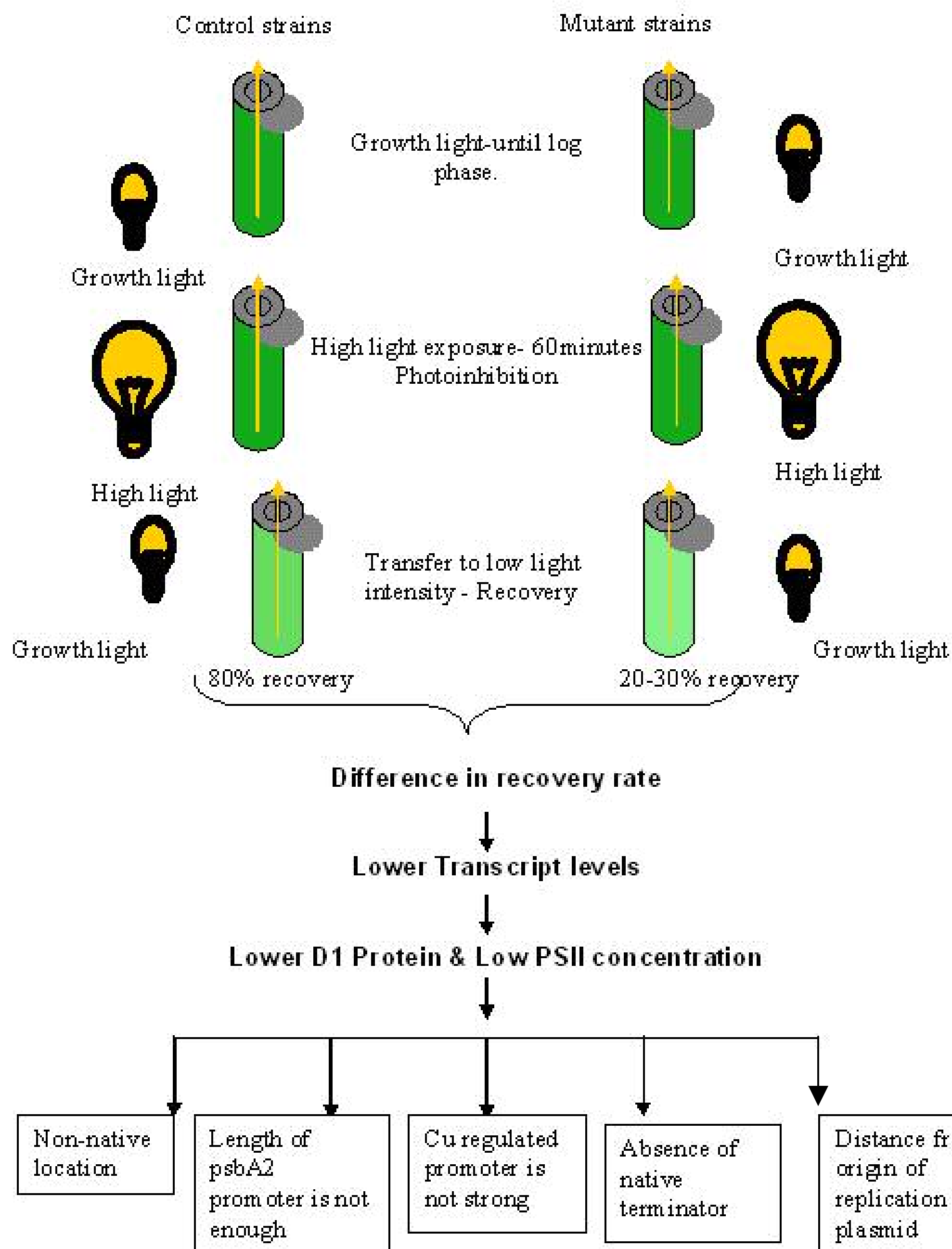


Figure 4.14: Flow chart of discussion

Conclusion:

It was required for the researchers to clone full length *psbA2* gene to study full length D1 protein including the N-terminus. They met with failure in expressing full length psbA2 gene in *E. coli*. We hypothesized that the N-terminus of D1 protein is involved in the turnover of D1 protein. To address our hypothesis we constructed MK1 and MK2 strains that allowed us to express the entire D1 protein including its N-terminus. While characterizing the strains, they showed light sensitivity due to low PSII concentration.

To address the lower PSII concentration in these strains, it was required to address rates of recovery of D1 protein in relation to *psbA2* transcript levels in MK1 and MK2 strains in comparison to WT strain. The recovery of D1 protein in MK1 and MK2 were low when compared to WT. The real time PCR results indicated the lower psbA2 transcript levels are responsible for the lower repair rates of MK1 and MK2 strains. The reason for low psbA2 transcript levels could be due to the position of *psbA2* gene in its non native chromosomal location and there is a potential for copper regulated promoter to be weaker than *psbA* promoter in *Synechocystis*. When I used *psbA2* promoter (-300 base pairs), the length of the promoter may not be adequate to drive the expression of D1 protein and the possibility of the absence of native terminator resulted in decreased *psbA2* levels and light sensitivity of strains.

I have provide a plausible explanation for low recovery rates in MK1 and MK2 are due to low level transcript levels making the strain prone to damage

even at low light intensities due to lower repair capacity (limitation due to transcript abundance).

Given the fluorescence assay data and real time data support my explanation; I therefore propose that the level of transcript regulates the rate of repair.

Overall my experimental results elaborates the knowledge of factors limiting rate of repair of PS II protein D1, which helps to understand the turnover process in a better way, indicating that the transcript levels regulates the rate of repair.

Chapter-V

Conclusions and Future directions

Photosystem II is the main reaction center component of photosynthesis consisting of many different cofactors and coenzymes of electron transport chain. D1 protein is the key protein of photosystem II. It combines the components of charge separation components in PSII. High light damages the D1 protein resulting in loss of PS II activity through photoinhibition (Mulo et al., 1998). D1protein is encoded by three psbA genes namely *psbA1*, *psbA2* and *psbA3* in *Synechocystis* sp. PCC 6803. Out of these three psbA genes, *psbA2* codes for 90% of D1 protein (Mohamed et al., 1993).

The high light induced damage to photosystem II D1 protein is called photoinhibition. Above certain threshold the rate of damage is directly proportional to the light intensity. This high light intensity induces damage to the D1 protein through oxidative damage or inactivation of electron transport (Aro et al., 1993), although the precise mechanism has yet to be established. There are different hypothesis on the mechanism of photoinhibition. The exact mechanism of photosystem II inactivation *in vivo* still needs to be elucidated (Mulo et al., 1998). On the whole I can explain that the PS II activity at any light intensity is a result of balance between the rate of damage and the rate of repair(Constant et al., 2000).

The damage to PS II leads to decrease in activity of photosystem II. To maintain the active role of PS II the damage need to be repaired. The D1 protein is synthesized co-translationally into damaged PSII complex. To accept newly synthesized protein, an empty slot for D1 should be available in PSII. The damaged D1 protein in PSII has to be degraded for the insertion of new D1 synthesis. Therefore the rate of degradation of the damaged D1 protein is oblig ately coupled with the insertion of newly synthesised D1 protein. Proteases are involved in the degradation of damaged. FtsH is the main protease responsible for the degradation of damaged D1 protein (Peter J. Nixon et al, 2004). The need of N-terminus for the action of FtsH protease was determined in *E. coli* by Chiba et al., 2002.

To determine the role of N-terminus in the repair process of D1 protein I constructed mutants called MK1 and MK2 with *psbA2* gene under all three *psbA* genes deletion background called 4E-3 strain as explained in chapter-III (Fig-3.1, Fig-3.2). During characterizing these mutants showed lower PSII concentration. This led to think about *psbA2* transcript levels in these strains.

To address my hypothesis that the transcript levels limit the rate of repair photoinhibition and recovery fluorescence experiments were conducted along with real time PCR. The cultures were photoinhibited at high light intensity of 1000 μmol m^{-2} s^{-1} for control strains WT, WT* and 800 μmol m^{-2} s^{-1} light intensity for mutants MK1 and MK2 to expose all four strains to same extent of damage. After photoinhibition the cultures were transferred to 20 μmol m^{-2} s^{-1} to allow the cultures to recover their active PS II, as explained in chapter II and chapter IV.

The experimental results indicated the recovery rate in mutant strains MK1 and MK2 are lower than the recovery rates of control strains.

To address the levels of transcripts for lower recovery rates of mutant strains MK1 and MK2, mRNA was isolated and used for quantitative real time PCR to determine the levels of transcript before photoinhibition(steady state) and one hour after photoinhibition (during recovery).

The transcript levels in control and mutant strains showed close proportionality with the recovery rates. The lower transcript level in MK2 and MK1 can be due to the position of *psbA2* gene in its non native chromosomal location and there is a potential for Copper regulated promoter to be weaker than *psbA* promoter in *Synechocystis*. When I used psbA2 promoter (300 base pairs) the length of the promoter may not be adequate to drive the expression of D1 protein and the possibility of the absence of native terminator resulted in decreased *psbA2* levels and light sensitivity of strains.

In this thesis my experimental results elaborates the knowledge of factors limiting rate of repair of PS II protein D1. The results demonstrate the relation between the rate of repair and the transcript levels indicating the rate of repair is proportional to the transcript levels.

Future directions of the project:

- Pulse-chase experiment:
    - To observe the *in vivo* relation of degradation of D1 protein and synthesis of D1 protein (turnover of D1 protein).

- Construction of new strains with various lengths of *psbA2* promoter and native terminator and change of neutral site region:
    - To identify the length of promoter for best expression
    - To identify the role of native terminator in the expression.
    - To determine the effect of location
- Construction of FtsH mutations in MK1 and MK2 strains:
    - To observe the effect of damaged D1 protein on the recovery.

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VITA

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Thesis: ECTOPIC EXPRESSION OF FULL LENGTH D1 PROTEIN AND FACTORS LIMITING PHOTOSYSTEM II (PSII) REPAIR IN *SYNECHOCYSTIS* sp. PCC6803.

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The research was originally conceived as a study on the role of N-terminus of D1 protein of the photosystem II complex. It was hypothesized that the N-terminus is required for the turnover of D1 protein, which is a repair process that maintains photosynthetic function. A *Synechocystis* PCC6803 mutant was constructed that allows us to study N-terminal mutation to test our hypothesis that the exposed N-terminus is needed for the action of FtsH protease in the process of degradation of the damaged D1 protein in Photosystem II repair process (Chiba et al., 2000, EMBO Reports 1, 47–52). During the course of these experiments, we observed that the constructs displayed impair D1 repair even with the wild type amino acid sequence. This led us to test the hypothesis, that the transcript levels restrict the rate of repair. To test of this hypothesis is the major part of the thesis project. The experimental data suggests that the rate of repair of D1 protein is proportional to the *psbA* transcript levels.

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