DEFINING THE PHOTOSYSTEM II REPAIR REGULON IN *SYNECHOCYSTIS* sp. PCC6803

By

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1998

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2006

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ACKNOWLEDGEMENTS

I express my sincere gratitude to my advisor Dr. Robert Burnap, for his continuous support and constructive guidance throughout my research program. I express my appreciation for his encouragement, expertise and for making me understand the need to be persistent to achieve any goal.

I extend my appreciation to my other committee members, Dr. Robert Miller and Dr. Rolf Prade, for their time, advice and assistance. I thank Dr. Patricia Ayoubi for her time, expertise and assistance with the microarray work. I express my gratitude to Dr. Louis Sherman our collaborator for contributing the mutant strain for this work. I thank the department of Microbiology and Molecular genetics for providing the financial support. A special appreciation to Dr. Hong Jin Wang , Shawn Daley and all other members of Dr. Burnap's lab past and present for their help in my research as well as for creating an enjoyable and pleasant work environment.

Finally I thank my parents and my husband Manoj for their unconditional support and encouragement to pursue my interests and for believing in me.

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NOMENCLATURE

1. mM ..... milli molar
2. µl ..... microlitre
3. ml ..... milliliter
4. DNA ..... deoxyribonucleic acid
5. RNA ..... ribonucleic acid
6. α ..... alpha
7. β ..... beta
8. γ ..... gamma
9. PCR ..... polymerase chain reaction
10. SDS ..... sodium dodecyl sulfate
11. cDNA ..... complementary DNA
12. LL ..... low light
13. HL ..... high light
14. PSI ..... photosystem I
15. PSII ..... photosystem II
16. SigD ..... sigma factor D

CHAPTER I

INTRODUCTION

The rate of free energy captured by photosynthesis using solar energy is 10^{17} kcal per year by photosynthetic organisms. This is ten times more than the fossil fuel energy consumed world wide per year. Oxygenic photosynthesis is driven by light, but one of its repercussions is formation of reactive oxygen species (ROS). The ROS can be scavenged by various cellular enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. These enzymes break down toxic derivatives of oxygen into water. Despite the presence of these enzymes, ROS is still capable of damaging proteins, nucleic acids, lipids thereby affecting various cellular components or processes. In plants or photosynthetic bacteria, photosystem II (PSII) located in the thylakoid membrane, is highly susceptible to this damage.

Damage occurs as a result of the formation of singlet oxygen species from triplet chlorophyll molecules derived from charge recombination within the PSII. The PSII complex is made up of more than 25 different proteins but the core of PSII comprises the D1 protein that harbors a Mn_4-Ca oxygen evolving complex, cofactors or complexes and chlorophyll molecules involved in electron transport chain through the PSII complex. The main target of oxidative damage is the D1 protein since it plays an essential role in electron transport and oxygen evolution (Nixon et al., 2005)

Light-induced damage primarily affects the D1 protein, although other subunits of PSII may occasionally sustain damage (Nixon et al., 2005) . The D1 protein is capable of undergoing degradation and rapid synthesis in response to light. The D1 repair cycle has been proposed to involve removal of the damaged the D1 subunit and insertion of newly synthesized D1 into the PSII complex in a mechanism that prevents the accumulation of damaged D1 within PSII complex (Nixon et al., 2005). Recently much of the research is mainly focused on understanding the mechanism involved in D1 turnover and the different proteases and transcription regulators, including the sigma factors, involved in this repair process. My project was aimed at understanding the different factors involved in repair of the D1 protein.

High light induced stress activates variety genes such as *psbA2*, *psbA3, ftsH (slr1604)*[1], ftsH *(slr0228)*, *sigD*, chaperones, and heat shock proteins. Sigma factor D (SigD) has been reported to be involved in the regulation of *psbA2* under high light (Imamura et al., 2003b). Does SigD control a repair regulon in *Synechocystis* sp. PCC6803? This simple sounding question forms the focal point of my research. High light stress and redox stress are closely related stresses in photosynthesis. High light results in the imbalance of electron flow as well as the generation of potentially damaging triplet states of chlorophyll. These factors combined with the abundance of oxygen produced by photosynthesis, results in the formation of reactive oxygen species (ROS).

Redox stress can be induced by light or by the addition of inhibitors such as 3-(3', 4' - dichlorphenyl) - 1, 1 – dimethylurea (DCMU) or 2, 5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB). These inhibitors are known to block the electron flow

[1] Designation refer to the original open reading frame annotations of *Synechocystis* sp. PCC6803 sequencing project

at key points in the electron transport chain (Imamura et al., 2003a). This leads to oxidative damage that has similar effects to photoinhibition. Redox stress also induces the expression of *sigD, psbA3, psbA2, ftsH (slr1604), ftsH (slr0228)* (I postulate that the products of these genes are a part of the photosynthetic repair system).

In this thesis I have also tried to understand the PSII repair regulon in redox stress, not in the presence of electron inhibitors, but by the action of thiol reducing agent such as dithiothreitol (DTT) in *Synechocytis* sp. PCC6803. DTT is a thiol reductant, capable of breaking disulphide bridges. It emulates the action of thioredoxin, a protein disulphide oxido-reductase (Balmer et al., 2003). The technique employed in this study to understand the different aspects of repair mechanism was global gene expression profiling. DNA microarrays are used as an efficient tool to study the expression profiles in *Synechocystis* sp. PCC6803 under different experimental conditions.

CHAPTER II

REVIEW OF LITERATURE

Cyanobacteria

Cyanobacteria are aquatic, photosynthetic bacteria that were previously mis-classified as “blue green algae”. Fossil evidence has revealed that cyanobacteria are more likely than 3.5 million years old (Vermaas, 2001). Few of the cyanobacterial groups are able to fix atmospheric nitrogen. The most commonly inhabited regions by these bacteria are liminic and marine environments. They thrive in salty, brackish and freshwater lakes or ponds. A variety of species are also found in hot or cold springs. Some species are halotolerant, capable of tolerating high saline concentration (Mur, 1999). Chloroplasts in plants are likely to have originated from these bacteria by the process of endosymbiosis and genetic integration.

The oxygenic atmosphere of earth today is by in large measure due to these cyanobacteria. The ability to perform both cellular respiration and oxygenic photosynthesis is by itself a unique characteristic of cyanobacteria. In contrast to plants and algae, cyanobacteria do not have specialized subcellular organelles to carry out photosynthesis or respiration. The cytoplasmic membrane of cyanobacteria separates the cytoplasm from periplasm and it harbors respiratory electron transport chain. By contrast photosynthetic electron transport system occurs in the thylakoid membrane is typically a multi-layered membrane system that extends throughout the cytoplasm.

The carotenoids pigments that are yellow in color are most abundant in the cytoplasmic membrane but are also contained in the membrane-protein complexes of the photosystems. Carotenoids play an important role in photoprotection as they are involved in dissipation of excess light energy and quenching dangerous triplet states of chlorophyll (Rakhimberdieva et al., 2004). The chlorophyll pigments, that are green in color are responsible for photosynthesis reside in the thylakoid membrane (Vermaas, 2001).

Photosynthesis

Photosynthesis is a complex physico-chemical process by which plants, algae and photosynthetic bacteria convert light energy into chemical energy. Oxygenic photosynthesis involves the liberation of molecular oxygen by using carbon dioxide from the atmosphere and water as an electron source to synthesize carbohydrates. Certain bacteria like the purple bacteria use light energy to synthesize carbohydrates but do not produce molecular oxygen, since they are incapable of using water as a source of electrons. The source of electrons for non-oxygenic photosynthetic bacteria may be hydrogen sulphide or hydrogen gas or reduced organic compounds. This process is termed as anoxygenic photosynthesis (Whitmarsh J, 1995).

Photosynthesis provides the energy, reduced carbon and molecular oxygen that are essential components of the earth's biosphere. The fossil fuels that are common utilized as the main source of energy for various human activities are the residual products of ancient photosynthetic organisms. Oxygenic photosynthesis, localized within cells or organelles of photosynthetic organisms, has a profound impact on the earth's atmosphere and climatic condition. Global photosynthetic fluxes are massive with more than 10% of the total atmospheric carbon being utilized each year by photosynthetic

organisms to synthesize carbohydrates. Most of this carbon is returned to the atmosphere as carbon dioxide as a byproduct in microbial, plant and animal metabolism.To understand the physio-chemical process of photosynthesis becomes imperative if we are to comprehend the relationship between living organisms and the atmosphere (Whitmarsh J, 1995).

The process of oxygenic photosynthesis is efficiently studied using cyanobacteria because they are amenable to rapid genetic manipulation and have a simple structure and fewer components than in plants. The core components of the photosynthetic electron transport chain in cyanobacteria are very similar to that found in plants. Plants contain chlorophyll 'a' and chlorophyll 'b', but cyanobacteria contain only chlorophyll 'a'. Plants utilize chlorophyll 'b' as a part of their light-harvesting antennae, whereas cyanobacteria utilize phycobilin in this role. Apart from this difference, the basic components of the photosynthetic mechanism are virtually identical in both the groups.

The thylakoid membrane cluster docks the two reaction centers namely photosystem I (PSI) and photosystem II (PSII). Upon light activation, the photons are used to split water and liberate oxygen and protons. Chlorophyll *a* (P680) in PSII absorbs the light energy, in the process an electron is boosted up to higher energy state. This electron passes through a series of electron carriers, beginning with reduction of the PQ (plastoquinone) pool. Then the electron is transferred from intra membranous PQ complex to cytochrome b_6f. The electron is then transported from cytochrome b_6f to PSI via a soluble electron carrier plastocyanin there by reducing the oxidized PSI reaction center. The chlorophyll *a* (P700) in PSI also absorbs the photons, becomes excited and loses an electron giving rise to an electron "hole" at the luminal face of PSI. This electron

is further transferred to ferrodoxin (Fd) that reduces NADP to NADPH that is eventually used to fix carbon dioxide to carbohydrates. This process of photosynthetic electron flow generates a proton gradient across thylakoid membrane that is used for generating ATP by utilizing ATP synthase (Vermaas, 2001).

Synechocystis sp. PCC 6803

Synechocystis sp.PCC6803 is the strain that is widely used by scientists around the world to study different aspects of photosynthesis. It serves as an excellent model for studying photosynthesis because *Synechocystis* sp.PCC6803 is easily transformable. The PSII complex in *Synechocystis* sp. PCC6803 structurally and functionally resembles the PSII complex of chloroplasts in plants and algae. The entire genome of *Synechocystis* sp.PCC6803 has been completely sequenced (Kaneko et al., 1996a, b). *Synechocystis* sp.PCC6803 can also grow photoheterotrophically using glucose as a sole source of carbon.

Photosystem II (PSII) complex

The most important reaction center for oxygenic photosynthesis is Photosystem II (PSII), this is a membrane bound complex responsible for utilizing water as a terminal electron donor. By using water as a source of electrons, PSII provides an abundant supply of reductant for biosynthetic metabolism. Photosystem II is found in all oxygenic photosynthetic bacteria, plants and algae (Barber, 2003). PSII consists of over 26 intrinsic and extrinsic membrane proteins, binding various pigments, metals and cofactors. However, the core of PSII consists of two structurally related polypeptides, D1 and D2. The D1-D2 heterodimer at the core of PSII binds the key cofactors involved in the initial

light-induced charge separation events leading to the oxidation of water and the subsequent reduction of quinones. The chlorophyll *'a'* molecules that comprise the primary electron donor, P680, is found between these proteins. Figure 1.1 represents the electron transport in photosystem II, also depicted are the numerous cofactors and coenzymes involved in this process of water oxidation and plastoquinone reduction.

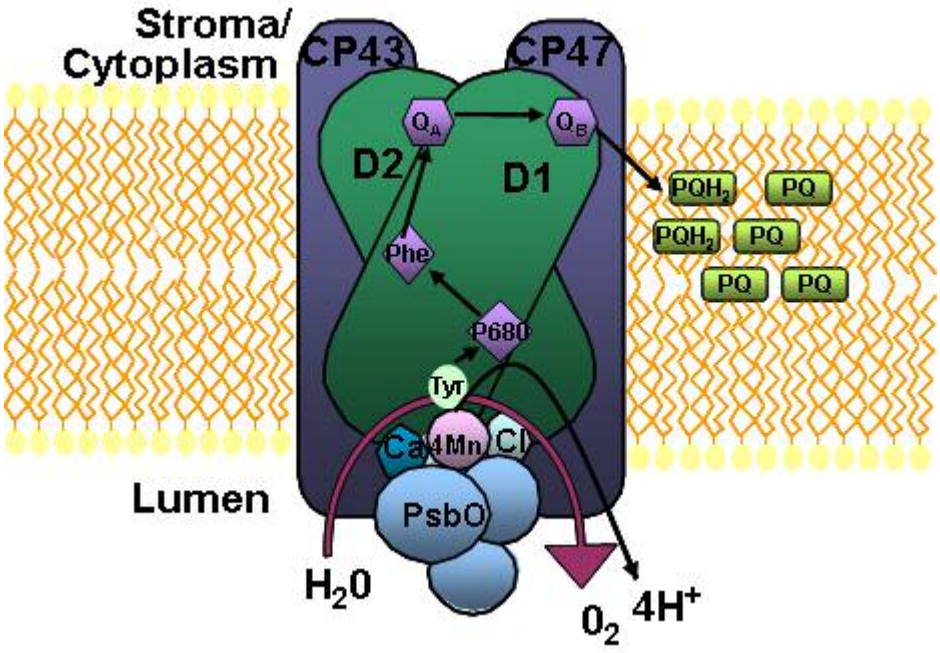

Figure 1.1: Electron transport pathway within PSII. The numerous subunits involved in the electron transport within the PSII are represented. The figure drawn using the data from the source (Pakrasi, 2004).

Photoinhibition

The photochemical reaction center of PSII is inactivated when photosynthetic organisms are exposed to high light conditions this process is termed as photoinhibition (Allakhverdiev et al., 2005). During the process of photoinhibition, the D1 protein, one of the heterodimeric proteins that constitute the reaction center of PSII, undergoes a structural modification corresponding to its irreversible damage. The precise nature of

this damage remains to be determined, although it is generally believed to involve the reactive oxygen species (ROS). Typically PSII is repaired by replacing the damaged D1 protein with the newly synthesized D1 protein. Damage and replacement of the D2 protein also occurs, but to a lesser extent than D1. The initial process involved in the repair mechanism is the degradation of the D1 protein in the photoinhibited PSII complex. This process begins by proteolysis of D1 by two proteases namely DegP2 and FtsH. Simultaneously the precursor D1 (pre-D1 or pD1) synthesized by *de novo* synthesis by the membrane–bound ribosomes, is integrated into the PSII complex. The integration of the precursor D1 into the PSII is a complex multi-step process. The initial step begins with *psbA* mRNA forming a complex with the ribosomes in the cytosol, the complex is targeted to the thylakoid membrane via a signal recognition mechanism. The elongating pre-D1 is inserted cotranslationally into the thylakoid membrane where it forms a complex with D2 protein. This complex interacts with other PSII subunits although details remain poorly understood. The pre-D1 is processed to yield mature a D1 protein and the Mn ions are assembled leading to the formation of the water oxidation complex, PSII subsequently regains its dimeric form (Allakhverdiev et al., 2005).

Importantly, the repair process appears to involve not only the synthesis and assembly factors, but also factors involved in the degradation of the damaged D1. Additional factors are presently being identified, one such factor is the FtsH which is a crucial membrane-bound protease that is required for efficient removal of damaged D1. Other factors, such as chaperonins and proteins absorbing released chlorophylls are also likely to be found as a part D1 repair mechanism. Transcripts for the D1 protein and known repair factors are up-regulated under conditions that lead to photoinhibition (eg.

high light). As discussed in the following sections, the goal of my research has been to better define the mechanism involved in regulating this repair processes. One potentially important candidate to exert a broad regulating influence over a set of PSII repair genes are the sigma factors. Of all the sigma factors known in *Synechocystis* sp.PCC6803, SigD seems to play a crucial role in the regulation of the *psbA* genes that encode the D1 protein (Imamura et al., 2003a). The proposed mechanism of D1 repair is illustrated in the Figure 1.2. Whether SigD is involved in the transcription of *ftsH* proteases and other factors in the repair process is biological problem under study.

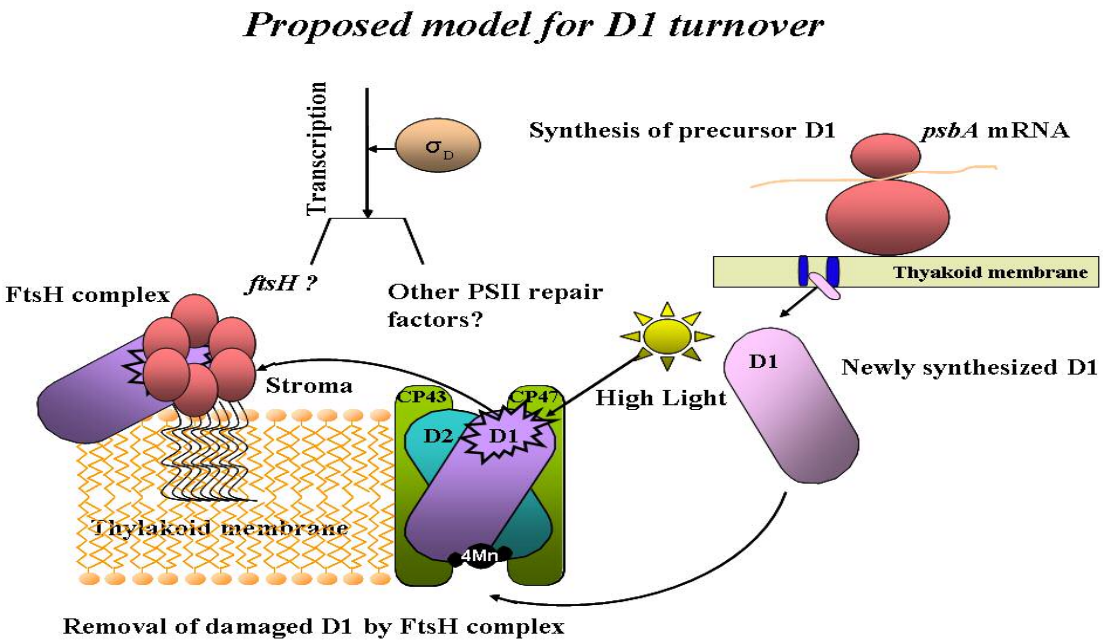

Figure 1.2: Proposed Model for D1 turnover. High light stress damages the D1, this damage sends a signal to recruit FtsH proteases to remove the damaged D1, the *psbA* mRNA synthesizes the nascent D1 that is co-translationally inserted into PSII complex. SigD is involved in the transcription of *psbA* genes but does it also play a role in the transcription of *ftsH* and other repair proteins? This is the biological question under study.

Role of FtsH proteases in D1 repair

The figure 1.2 summarizes the proposed model for D1 repair in *Synechocystis* sp. PCC6803. Light induces photodamage on PSII causing impairment in the electron transport chain, subsequently leading to a conformationl change in the reaction center proteins mainly D1, driving the recruitment of proteases via a signal recognition particle. The heterodimeric PSII subunits monomerize, followed by partial disassembly of PSII complex. The damaged D1 is removed, degraded and newly synthesized D1 is reinserted in to the PSII complex. This is followed by light activated (photoactivation) reassembly of Mn_4-Ca cluster and the various extrinsic proteins. This photoactivation is said to be triggered only after proteolytic processing of the C-terminal of the D1 protein (Nixon et al., 2005).

Currently, research is mainly focused on identification of different proteases involved in the process of D1 turnover. The proteins identified to be involved in the repair cycle are DegP2 and FtsH proteases in the chloroplast of plants whereas so far only FtsH is observed to be solely responsible for D1 turnover in *Synechocystis* sp. PCC6803. FtsH is a membrane-bound metallo-protease consists of Zn^{2+} -containing catalytic site responsible for the proteolytic activity and an AAA^{+} domain (ATPase associated with various cellular activities). Additionally it may also contain a C-terminal leucine zipper motif. There are four FtsH proteases in *Synechocystis* sp. PCC6803 namely Slr1390, Slr1604, Slr0228, Sll1463. The two FtsH proteases required for cell viability are Slr1604 and Slr1390.

The FtsH proteases proposed to be responsible for “photoprotection” (removal of damaged D1) in *Synechocystis* sp. PCC6803 are Slr0228, Slr1604. The former was

confirmed to have this role with studies using insertional mutants of *slr0228* (Nixon et al., 2005). The FtsH protease Slr0228 in *Synechocystis* sp. PCC 6803 is also said to be involved in the removal of unassembled PSII subunits and complexes from thylakoid membrane (Komenda et al., 2006).

The proposed model for FtsH mediated D1 cleavage is that the N- terminus of the damaged D1 is exposed to the stroma, due to a conformational change occurring due to the cleavage in the Q_B- binding site (Nixon et al., 2005). FtsH is said to be located in the stromal regions. The exposure of the N-terminal portion of D1 initiates the proteolytic cleavage by FtsH. It has also been suggested for initiation of FtsH cleavage at least twenty amino acid residues must be exposed, and the target protein must be unfolded. FtsH is also likely to contain transmembrane helices that interact with membrane proteins. Slower D1 degradation rates were observed in FtsH mutants (Nixon et al., 2005).

Role of High Light Inducible Polypeptides (HLIPs) in PSII repair

There are four high light inducible proteins in *Synechocytis* sp. PCC6803 that are designated as HliA, HliB, HliC and HliD. All four polypeptides were shown to be accumulated during high light (HL) exposure, low temperature or nitrogen or sulphur starvation (He et al., 2001). Studies using null mutants of all four highlight inducible genes showed that these mutants are unable to adapt to high light (He et al., 2001). These mutants grow well in low light intensity (LL) as the wild-type (WT) but lose their capacity to photosynthesize when exposed to high light. They are unable to adapt to high light.

A similar group of proteins that tend to accumulate when exposed to high light are the ELIPs (early light inducible proteins). This was observed in plants namely seedling of pea and barley (He et al., 2001). ELIPs and HLIPs belong to the same family of proteins and are accumulated in variety of stress conditions which indicates that these proteins are likely to play an important role in either photoprotection or repair during photoinhibition (He et al., 2001). For the present discussion the terms HLIP and ELIP are considered synonymous.

HLIPs belong to the family of chlorophyll binding proteins light harvesting complexes (LHC) super family of proteins. Sequence analysis of HLIPs has revealed that they are comprised of conserved residues that are capable of binding to free chlorophyll molecules (He et al., 2001). The effects of high light stress are formation of reactive oxygen species and charged chlorophyll molecules ("singlet excited chlorophyll") that are potentially hazardous to the biological system. The involvement of HLIP in photoinhibition is not completely understood but it is proposed to act as "pigment carriers" thereby involved in transferring chlorophyll pigments to reaction centers as they are synthesized, and also involved in the turnover of PSII components during photoinhibition. Hli polypeptides are very similar to each other, HliA and HliB share 87.1% sequence identity whereas HliC and HliD share only 44.7% sequence identity. Another interesting aspect of these proteins is that they hardly seems to accumulate in LL, since *hli* genes are induced only during HL or redox stress. It is not completely understood whether these proteins are involved in binding to free chlorophyll or dissipation of excess absorbed energy (He et al., 2001). Studies have shown that *Synechocystis* sp.PCC6803 contains five of these genes that are homologs of eukaryotic

chlorophyll *a/b* binding proteins. The proteins are called as small Cab-like proteins (SPC), the *spc* genes are 174-213 bp in length and the proteins are presumed to have single transmembrane helix. The sequence analysis indicates that the SPC proteins have high similarity with HliA from *Synechococcus* sp. PCC7942. SPC proteins are involved in binding to the free chlorophyll and thereby may play a key role in turnover. They may also be involved in transporting the chlorophyll molecules to reaction centers and antenna complexes from the sites of synthesis. The authors assume that HliA could also play a similar role in high light or redox stress in transporting free chlorophyll molecules to prevent oxidative damage (Funk and Vermaas, 1999).

Sigma factors

The eubacteria RNA polymerase (RNAP) is a holoenzyme and it consists of the following subunits α_2, β, β', ω and σ. The core of RNA polymerase is responsible for polymerase activity and the σ is involved in the recognition of the promoter sequence on the DNA template and directing the RNA polymerase to initiate transcription. The cyanobacterial RNA polymerase also consists of α_2, β, β' subunits, but β' subunit is split into two parts γ ($RpoC_1$) and β' ($RpoC_2$). Hence the cyanobacterial RNA polymerase consist of α_2 β β'γ and σ (Hansen, 2004). The promoters are called 'consensus promoter' because they contain the typical recognition sequences such as 'TATAAT' at the -10 position and 'TTGACA' at the -35 position in relation to the transcription initiation site (Goto-Seki et al., 1999).

Bacteria are capable of synthesizing several sigma factors, each of these sigma factors are able to recognize a unique set of promoters (Hansen, 2004). The sigma factors are activated in response to the corresponding environmental or internal physiological

condition. As mentioned earlier the sigma factors are capable of recognizing a specific set of promoters to coordinate cellular process.

The sigma factors have been classified into two broad families based on their structural and functional similarities.

1. σ^{70} – Sigma factors having molecular weight of about 70kDa
2. σ^{54}- Sigma factors having molecular weight of about 54kDa

The σ^{70} family has been further divided into three groups -group I, group II and group III.

The group I sigma factors

This group includes the primary sigma factor (PSF) designated as sigma factor A (SigA). These sigma factors are responsible for the expression of genes required during the exponential growth phase. SigA regulates transcription of various house keeping promoters, and is essential for cell viability.

The group II sigma factors

This group comprises PSF-like sigma factors and that includes SigB, SigC, SigD and SigE. These sigma factors closely resemble the primary sigma factor based on their amino acid similarities and depending on their type enable cyanobacteria to adapt under stress conditions such as high light, long term starvation of nitrogen, carbon or sulphur. In some species a PSF-like sigma factors also affects the setting of circadian clock, but most of its physiological functions are still unknown. The SigB and SigC are said to be involved in transcription of genes during carbon and nitrogen stress, and are not essential for cell viability (Hansen, 2004).

The group III sigma factors

Polypeptides that belong to this group are termed as alternative sigma factors and comprises of SigF, SigG, SigH, Sig I. These proteins are structurally different from both group I and group II sigma factors. Mainly involved in transcription of genes during "extracytoplasmic stress conditions"(ECF) in other words these sigma factors control expression of genes whose products function in the periplasm, cell membrane or extracellular environments such as chaperones, proteases, thio:sulphide oxidoreductases, sporulation, synthesis of flagella and heat shock response, they essentially replace the primary sigma factor under unfavorable growth conditions.

The σ^{54} family of sigma factors is structurally different from σ^{70} family. These proteins are less abundant and none of its members are found in actinobacteria and cyanobacteria genomes. The sigma factors in this group are thought to be involved in the regulation of nitrogen assimilatory pathways, flagella synthesis and nitrogen fixation in certain bacteria (Hansen, 2004).

The group II sigma factors include SigB, SigC, SigD and SigE exhibit high sequence similarity. That is indicated in the Figure1.3, unrooted tree structure comparing the sequence similarity between the various sigma factors in *Synechocystis* sp. PCC6803. As observed from Figure 1.3, SigD is structurally related to group II sigma factors namely SigB, SigC and SigE, whereas it is distantly related to the sigma factors in group I and group III. Figure 1.4 shows the close resemblance between the group II sigma factors based on the amino acid sequence.

Figure 1.3: Sequence similarity between the sigma factors in *Synechocystis* sp. PCC6803**.** The group II sigma factors have a high sequence similarity and this observed as the branches between SigB, SigD and SigC are very close to one another

```
sll0306-SigB SYLHEIGRVPLLTHEQEIILGRQVQQMMALLEHRRALAD--------------------R
sll2012-SigD AYLREIGRVPLLTHEEEIVYARQIQQVVSLNEIRRSLAE--------------------G
sll0184-SigC LYLQDIGKIPLLRRDEEVHIAQQVQSYLRLVEIQNRAAESDAAIDQYQTAIAVHDQLLVQ
sll1689-SigE AFFREMARYPLLSAAEEVELARQIRLLVSAEDVRQQLTQ--------------------Q

sll0306-SigB LGREP----------------------SDP-EWAEAADLS-VTRLHRYLGQGERARRRMIE
sll2012-SigD KDGEP----------------------VSPSEWARAADLS-IRELERAIREGERARRRMVE
sll0184-SigC LGHRPSYERWARILGQTVATLRQTLRSGKRRWAELAGLT-VEELENIERQGITARAHMIR
sll1689-SigE LERTP----------------------SLQEWGQALEFPQVRQFEIWLYQLRAARRRMIR

sll0306-SigB ANLRLVVAIARRYQRRNMEFLDLIQEGSLGLERGVERFDPTRGYRFSTYAYWWIRQAITR
sll2012-SigD ANLRLVVSVARRYLRRNLDLLDLIQEGTIGMQRGVERFDPTRGYRFSTYAYWWIRQAITR
sll0184-SigC ANLRLVVSVARRYQNRGLELLDLIQEGTLGLERAVERFDPTRGYRFSTYSYWWIRQGITR
sll1689-SigE SNLRLVVSIARRYLNRGVPFLDLIQEGAIGLMRAAERFDPDRGYRFSTYAYWWIRQAITR

sll0306-SigB AIAQQGRTIRLPIHITERLNRIKRTQRELSQQLGRSATPAEVARALEIDPSQIREYLSLS
sll2012-SigD AIAERSRTIRLPIHITERLNRIRRAQRQLSQERGRAASIAELAEHLELTPRQVREYLERS
sll0184-SigC AIATQSRMIRLPVHITERLNRIRRAQRRISQERGHTPRIDEVAEELGMTPEQVREVLTQV
sll1689-SigE TIANDARTIRLPIHVVERLNRIRRAQRSLRQELRRNPNEGELAAAALDITPAQLRQLLQLR

sll0306-SigB RQPISLDVRVGDNQDTELSELLEDE-GVSPDAYITQESMRQDLQNLLAE-LTPQQQAVLT
sll2012-SigD RHPLSLDLRVGDNQDTELGDLLEDD-GPLPEDFATYASLQLDLDSLMAE-LTPQQREVLI
sll0184-SigC PRSVSLELRVGQDRDTELMDLLETD-TQSPEDELMREALQNDMQEILLD-LTPREQEVIA
sll1689-SigE RQSLSLNHRVGRGEDTELVDLLEDQQLQLPEDLMNESMLRREIVEVLAEVLTEREMEVIC

sll0306-SigB MRFGLNDGQELSLARIGQHLNISRERVRQLENQALAQLR--RRRANMAEYIIAS-
sll2012-SigD LRFGLNDGQPLTLASIGSMLSISRERVRQIEREALNRLR--RRRSMIQEYLAS--
sll0184-SigC LRFGFQDGVAHSLSEIGRILNLSRERVRQIEARALQRLRHPRRRDRIRDYYENLG
sll1689-SigE LRYGIASHQSYTLEEVGNMFNLSRERVRQIQSRAMRRLRRPQVARRLRGWL----
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Figure 1.4: Amino acid sequence alignment of group II sigma factors. The sigma factors in this group include SigB, SigC, SigD and SigE, the green code indicates the conserved residues within this group.

Experimental background for sigma factor D

The *sigD* gene (*sll2012*) encodes for a 40kDa sigma factor D protein (SigD) in *Synechocystis* sp. PCC 6803. Understanding the function of cyanobacterial sigma factors under environmental stress is a topic of paramount interest. Limited information is currently available about light responsive sigma factors in photosynthetic bacteria. SigD is termed as "High-light responsive sigma factor" (Imamura et al., 2003b). Studies have indicated that SigD is increased to 3.4 times in high light than in normal light (Imamura et al., 2003b). None of the other sigma factors or the RNA polymerase core subunits seem to be elevated under same conditions. Earlier reports have revealed specific promoter recognition by *E.coli* RNA polymerase using cyanobacterial sigma factors (Imamura et al., 2003b). This was confirmed by conducting *in vitro* promoter recognition of *psbA2* by SigD using RNA polymerase reconstituted from *E.coli* core (Imamura et al., 2004). The transcriptional control of *psbA* encoding the D1 protein has been extensively studied. This was the first report connecting *psbA2* expression to a specific sigma factor (Imamura et al., 2003b)

This allows one to hypothesize that SigD plays a role in the repair mechanism of PSII under high light. Previous studies using cyanobacterium *Microcystis aeruginosa* K-81 have indicated that the principal sigma factor is capable of recognizing the K-81 *psbA2* promoter, also exhibits light responsive and circadian rhythm transcription (Imamura et al., 2003b).

Immunological evidence has shown increased levels of SigD when the cells were exposed to light after adaptation to darkness (Imamura et al., 2003a). The increase was two-and-one-half-fold and it persisted for nine hours. The amount of SigE was also

shown to be elevated but it gradually increased after three hours and reached a peak after ten hours. In contrast SigB decreased under light and only 25% remained after nine hours. SigB levels increased to about two-fold in dark whereas SigE decreased to about 65% when exposed to darkness for about nine hours. SigA and SigC remained constant under both light and dark conditions. These results indicate that SigD is rapidly induced in light whereas SigE is slow to respond to light, while SigB is induced in darkness (Imamura et al., 2003a).

Sigma factors are capable of regulating each other's transcription and this has been well documented in cyanobacterial strains (Lemeille et al., 2005a). It is also been suggested that *sigA* indirectly regulates the expression of *sigD* by transcribing a repressor of *sigD* (Lemeille et al., 2005a). Quantitative RT – PCR experiments using *sigD* deletion mutants have also shown that transcription of *sigA* and *sigB* genes decreased by ~two to three-fold. It was also observed that *sigE* mutation can cause a strong decrease (~ 20-fold) in the transcription of *sigA* and *sigB* genes and about three to five-fold decrease in the transcription of *sigC* and *sigD* genes. This study indicates that SigE is an important sigma factor and it controls the expression of the three *sig* genes (*sigB, sigC* and *sigD).* Mutations of *sigE* are capable of inactivating sigma factor genes mainly affecting the house keeping genes *sigA* (Lemeille et al., 2005b)

Microarray and Northern analysis have shown that *sigE* mutants decreased transcript abundance for the genes that are involved in the glycolysis, the oxidative pentose phosphate pathway and glycogen metabolism. This indicates that SigE is involved in coordinate regulation of a set genes or in other words SigE controls a regulon (Osanai et al., 2005).

The phylogenetic analysis of group 1 and group 2 sigma factors have indicated that there is similarity in the recognition domain for -10 regions because several amino acids residues that are responsible of the recognition of DNA bases are highly conserved. The -35 promoter regions are not as highly conserved as the -10 regions, this may be the reason for the promoter specificity of the different sigma factors (Goto-Seki et al., 1999).

The expression levels of SigB/SigD in the presence of drugs Rif (rifampin), Cm (chloroamphenicol), herbicides (DCMU, DBMIB) and red versus blue light were examined. The Rif is an inhibitor of translation, Cm is an inhibitor of transcription, DCMU is an inhibitor of electron transport between PSII and PQ and DBMIB is an inhibitor of electron transport between PQ and cytochrome b_6f complex. The electron transport inhibitors DCMU and DBMIB are often used to manipulate the redox poise of the photosynthetic electron transport chain that is hypothesized to control same gene expression, details provided in later sections. These herbicides block electron transport at specific points causing an accumulation of electrons. Western blot analysis has shown that SigB and SigD levels declined during the treatment with Cm but not by Rif under light implying that the induction may not be regulated at the level of transcription (Imamura et al., 2003a). In the presence of DBMIB, the level of SigB was found to be significantly increased. SigB synthesis was induced due to the redox condition where the electron transport chain is reduced upstream of b_6f complex and oxidized downstream of PQ pool. In contrast SigD was elevated when cells were treated with DCMU and light. The *sigD* response is induced due to the reductive state of the components upstream of the PQ pool (Imamura et al., 2003a). Treatment with DCMU during small intervals of time increased the level of *sigD* transcripts but the amount of *sigD* transcripts remained

constant during normal growth conditions, heat shock, low temperature and salinity treatments (Tuominen et al., 2003).

These results were further confirmed by DNA microarray experiments using DCMU and DBMIB as electron inhibitors (Hihara et al., 2003). As mentioned earlier DCMU blocks electron flow from PSII to PQ pool, therefore the PQ pool remains oxidized. DBMIB blocks the flow of electrons from PQ to cytochrome b_6 and *f*, thereby rendering PQ pool reduced. Hence the two inhibitors have antagonistic effects on the net redox state of PQ pool.

The microarray data shows that addition of DCMU and DBMIB caused changes in gene expression but only few of the genes are described here. The data suggests that addition of DCMU caused an induction in the following genes such as *gifA*, c*hlB*. The addition of DBMIB induced some genes that were not seen to be induced by DCMU these include *dnaK*, *groESL*, *groESL-2*, *dnaJ*, *htpG*, *clpB*, *hspA*, *hliA*, *hliB*, *hik34*. This illustrates the changes in redox state induced by DCMU and DBMIB have different effects on gene expression. There was a subset of genes that was induced under both conditions such as *ftsH* (*slr1604*), *sigD*, *psbA3*. These data shows that *sigD*, *ftsH* (*slr1604*) and *psbA3* respond in the same manner as in HL. It is interesting to acknowledge that the redox state of the PQ pool did not affect transcription of photosynthesis related genes. The genes that were observed to be repressed with DCMU and DBMIB include genes encoding for ribosomal proteins, phycocyanin, ATP synthase, enzymes for biosynthesis of photosynthetic pigments, thioredoxin and nitrogen regulated genes. This data also indicates that redox regulated genes are a small subset of genes that

overlap with genes that are affected by high light. The changes in the transcript abundances in this redox experiment was similar to high light effect (Hihara et al., 2003).

SigD also exhibited enhanced expression in red-blue light as judged by northern and immunoblot assays (Imamura et al., 2003a). From all these results it has been suggested that a particular wavelength of light may be capable of modulating the redox state that caused the induction of SigD. The light induced expression of SigD was studied by using two representative genes namely *psbA2* (*slr1311*) and *psbA3* (*sll1867*). The amount of *psbA2* transcripts decreased drastically in *sigD* deletion mutants *(*Δ*sigD*) under light (Imamura et al., 2003a). The transcripts were reduced to about 50% of their initial level after three hours when compared to WT (wild-type) likewise with *psbA3*. These results are consistent with the hypothesis that SigD controls the expression of *psbA2* and *psbA3* under light. Studies suggest that the structural changes to SigD may be caused by redox signal along with other *trans* –acting factors such as the enhancers may boost the sigma binding affinity to the promoter or the core enzyme (Imamura et al., 2003a). Studies have also indicated that *sigD* transcripts are also reduced in ΔHik33 (a sensor histidine) (Imamura et al., 2003a) . This indicates that sensor protein may be involved in the expression of *sigD* (Imamura et al., 2003a).

Promoter selectivity of group 1 and group 2 sigma factors were examined using three genes *psbA2, hspA*, *lrtA*. The assay performed to analyze the promoters of these genes was i*n vitro* run-off transcription assay. The signal intensity from *psbA2* promoter was greater than *hspA* and *lrtA* promoters for each sigma factor, indicating that *psbA2* was stronger. Although it shows that the sigma factors are capable of recognizing *psbA2* but the efficiency in promoter recognition specificity between the various sigma factors

were not uniform. These differences in specificity may be due to the promoter architecture. They also studied the influence of -35 hexamer on promoter recognition by group 1 and group 2 sigma factors. They conducted *in vitro* analysis using SigA and SigD both have known to transcribe *psbA2* (Imamura et al., 2004) .

The *psbA2* promoters in the WT had both -10 and -35 hexamers but the promoters in the mutant had -10 hexamer intact, with AC-TG mutations at -35 hexamer. The results obtained indicated that -35 hexamer was essential for promoter recognition by SigA but not for SigD. Hence group 1 and group 2 sigma factors appear to have a differences in the requirement of conserved -35 regions (Imamura et al., 2004) .

As mentioned earlier *E.coli* β' (RpoC) in cyanobacteria is divided into two parts namely γ (RpoC1) and β' (RpoC2). *In vitro* studies using *E.coli* reconstituted RNA polymerase (Ec) and cyanobacterial sigma factors (σ^A, σ^C, σ^D) showed that transcription of *psbA2* was significantly increased in the presence of cyanobacterial RpoC2 and SigD ($Ec\sigma^D$ + RpoC2). No enhanced transcription was observed with the addition of RpoC1. None of the other sigma factors exhibited such an enhancement this suggests that RpoC2 plays an important role in accelerating the transcription of *psbA2* (Imamura et al., 2004).

Photosynthetic electron transport induced by light is an important factor in the transcriptional and post transcriptional regulations in photosynthetic bacteria, algae and plants. The redox state of electron transport chain can affect transcription, mRNA stability, splicing, translation and post translation modification (protein phosphorylation) in plants and algae. In cyanobacteria, changes in redox state mainly affect transcription and mRNA stability. Various genes are affected by redox regulation namely, *psbA* (encodes for reaction center D1 polypeptide in PSII), *psaE (*encodes a subunit of PSI),

cpcBA (encodes for α and β subunits of phycocyanin), *rbcLS* (encodes for ribulose 1,5 biphosphate carboxylase), *desA,-B,-D* (genes encoding fatty acid desaturases), *trxA* (gene encoding thiodoxin), along with some nitrogen and heat shock genes (Hihara et al., 2003).

Microarray analysis of *Synechocystis* sp.PCC6803 genome under iron deficient versus iron sufficient conditions have indicated that group 2 sigma factors *sigB, sigC* and *sigD* are up-regulated in iron deficient conditions (Singh et al., 2003). The *sigD* also exhibited ten-fold up-regulation under oxidative stress condition (presence of hydrogen peroxide, H_2O_2). Two genes *sll2013* and *sll2014* that are contiguous to *sigD* were also induced in the presence of H_2O_2 .The genes exhibited 4.8-fold and 3.3- fold induction respectively. Therefore, *sll2012-sll2013-sll2014* may form a transcriptional unit (Singh et al., 2004). Results from microarray analysis of inorganic carbon limitation studies have also indicated increased abundance of *sigD* transcript about 3.59-fold up-regulation at 200 minutes and this finding was consistent with the up-regulation of *psbA* genes despite the decreased expression of all other photosynthesis genes (Wang et al., 2004).

Microarray analysis of high light treatment in *Synechocystis* sp. PCC6803

Studies based on time-dependent gene expression in *Synechocystis* sp. PCC 6803 during acclimation to high light have been thoroughly investigated by various research groups (Hihara et al., 2001). A Japanese-based research group have investigated the response in gene expression following a shift from low light (light intensity = 20 μmol photons m^{-2} sec^{-1}) to high light (light intensity = 300 μmol photons m^{-2} sec^{-1}). The acclimation to high light was studied for different time intervals namely fifteen minutes, one hour, six hours and fifteen hours. The microarray results indicate down-regulation of

genes encoding for subunits of PSI, *heme* , *chl* , *apc* and *cpc* in order to avoid absorption of excess light energy. There was reduction in antenna size and also PSI subunits. Results also indicate genes responsible for uptake of carbon dioxide and carbon dioxide fixation were also up- regulated these include *ndh* genes (encode for high affinity CO_2 transporters), *ccm* genes and *rbc* genes (encode the subunits of ribulose biphosphate carboxylase/oxygenase). The *psbA* genes, glutathione peroxidase, *clpB*, *htpG*, *dnaK*, *hsp17*, *groESL*, *sodB*, were also up-regulated and these genes are involved in protection against oxidative stress caused due to high light. The *rpl* and *rps* genes were also up-regulated these genes are involved in the synthesis of 50S and 30S ribosomal subunits. This depicts there is an acceleration in protein synthesis because cells divide faster in high light (Hihara et al., 2001).

CHAPTER III

MATERIALS AND METHODS

Construction of pPETE*: sigD* plasmid vector

A plasmid cloning vector capable of replication in *E.coli* was constructed to contain the *sigD* gene encoding for Sigma factor D. As discussed later in this chapter the plasmid is also capable of integration into *Synechocystis* sp. PCC6803 chromosome where the *sigD* was under the control of a constitutively expressing petE promoter. The plasmid was constructed in the following manner. The *sigD* gene was amplified using a standard PCR reaction. The flanking region of the primers incorporated *Sap*I restriction sites in order to facilitate the sticky-end ligation into vector pPETE:*patA* low copy plasmid (constructed by Wang et al; unpublished) the *patA* gene was flanked *Sap*I restriction sites. The primers (table 2.1) that were used to construct amplify *sigD* the primers were engineered to integrate *Sap*I restriction sites.

Forward primer	$_{5'}$AATTAAGCTCTTCCATGACCAGAACCAGCC$_{3'}$
Reverse primer	$_{5'}$ATATATGCTCTTCGTTAGGCTAAATACTCC$_{3'}$

Table 2.1: The forward and reverse primer sequences used for the PCR amplification of *sigD.* The primers were engineered to incorporate *Sap*I restriction sites for cloning purposes.

PCR amplification of *sigD*

The 100µl reaction mix was prepared with the following components: 10 µl of 10X buffer (Invitrogen, USA), 2 µl of 50 mM magnesium sulfate (Invitrogen, USA), 1.5 µl of 10 mM dNTP mix, 5 µl of 20 µM primer pair, 0.5 µl of 1 µg/µl chromosomal DNA and the 0.8 µl of 2.5 U/µl Platinum *Pfx* DNA polymerase (Invitrogen, USA). The total volume was made up to 100 µl with double distilled water. The PCR conditions were (94°C 2 minutes, 94°C 30 seconds, 62°C 45 seconds, 68°C 3 minutes)-1°C/cycle for 10 cycles (94°C 30 seconds, 52°C 45 seconds, 68°C 3 minutes) X 25cycles, 68°C 20 minutes, 15°C hold. The PCR products were analyzed on 0.8% agarose gels. The PCR products were purified using Qiagen QIA quick PCR purification kit (Qiagen, USA) to a final elution volume of 40µl in double distilled water and again analyzed on 0.8% agarose gel.

Restriction enzyme digests

The purified insert was digested using restriction enzyme *Sap*I (New England Biolabs, USA). The restriction digest was set up using 16 µl of insert (PCR amplified *sigD* as described in previous section), 8 µl of 10X NE buffer 4 (New England Biolabs, USA) 8 µl of 2 U/µl *SapI* restriction enzyme. The total volume was made up to 80 µl with double distilled water. The reaction was conducted at 37°C water bath for four hours. The *Sap*I digested PCR products were purified by precipitation using one half volumes of 7.5 mM ammonium acetate and two and a half volumes of cold absolute ethanol. The ~7.2kb plasmid pPETE: *patA* was also digested using *Sap*I (New England Biolabs, USA), this vector contained two *Sap*I restriction sites that flank the *patA* gene, that was eventually replaced by *sigD* gene. The reaction was incubated for four hours for

complete digestion and the digested plasmid devoid of its former *patA* insert, was purified by preparative gel electrophoresis followed by extraction from the agrose gel using a Qiagen QiaexII gel extraction kit (Qiagen , USA).

Ligation

The vector and insert DNA fragments prepared as above were ready for ligation. The ligation was carried out using ~500 ng of purified *Sap*I digested plasmid vector and ~500 ng of *Sap*I digested and purified insert. The enzyme for the reaction was 2 µl of T4 DNA ligase (New England Biolabs). The ligation was carried out at 14°C in a water bath for eighteen hours. The nucleotides in the ligation mixture were purified by using ethanol precipitation with 2 µl of tRNA (10 µg/ml) as a co-precipitant.

Transformation into *Escherichia coli* XL-1 Blue Super Competent cells

Transformation was carried out using XL-1 Blue super competent cells (Stratagene, USA). The cells were thawed on ice and 50 µl of cells were added to the 0.5 ml prechilled Eppendorf tubes. Along with the cells, 0.9 µl of β-mecaptoethanol was added to the tube. The contents were mixed by swirling gently every two minutes while incubating on ice for 30 minutes. Heat pulse was applied by placing the tube in a water bath at 42°C for 45 sec. Then it was incubated again on ice for another two minutes. Finally the contents were transferred to 14 ml BD Falcon polypropylene tube that was prefilled with 0.9 ml preheated SOC medium (Russell, 2001). The tube was incubated at 37°C for one hour with constantly shaking at 225-250 rpm. The entire contents were plated on several Luria broth agar (Bactoagar, Difco) plates containing 30 µg/ml spectinomycin (Sigma-Aldrich, USA).

Isolation of recombinant plasmids

Single colonies were picked and streaked on fresh LB plates containing 30 µg/ml spectinomycin. The colonies were picked and grown in 2 ml LB with 30 µg/ml spec overnight at 37°C with constant shaking at 225-250 rpm. The plasmids were extracted and purified using alkaline mini preparations procedure (Russell, 2001). The plasmids were screened using the two restriction enzymes *Sal*I and *Bam*HI for the presence of the new *sigD* insert replacing the former *patA* insert.

In the desired recombinants *Bam*HI has two restriction sites one each in the vector and the insert whereas *Sal*I has only one restriction site in the vector. A master mix for each enzyme was prepared. The master mix for *Sal*I (New England Biolabs) contained 24 µl of the enzyme, 12 µl of 10 X buffer, 2.4 µl of BSA and 189.6 µl of nanopure water. This was enough for 11 reactions and each tube had 9 µl of the master mix. The master mix for *BamHI* (New England Biolabs) contained 24 µl of the enzyme, 12 µl of 10 X buffer and 192 µl of nanopure water. There were 11 restriction enzyme reactions conducted for each enzyme. About 1 µl of the mini prep DNA obtained from separate isolates was added to each of the 11 reaction tubes. The digestion was carried out at 37°C for one hour and the digests were confirmed on 0.8% agarose gels. The results for the restriction digestion are depicted in the figure 2.1. The uncut plasmid was 7.6 kb in length and the plasmid cut with BamH1 yielded two fragments 3.7 kb and 3.8 kb in length. The double digest also showed only two fragments on the gel because the two restriction sites of *Bam*H1 and *Sal*1 in the vector are in close proximity and they differ by 276 bp, hence there is a shift in the bands as observed in figure 2.1.The purified plasmid was prepared using Qiagen Midi Plasmid extraction kits (Qiagen, USA) and the plasmid was then sent

to the core facility (Oklahoma State University, Stillwater, OK) for sequencing. The plasmid map is illustrated in figure 2.2 indicates the restriction site used for inserting the genes and also for the analysis.

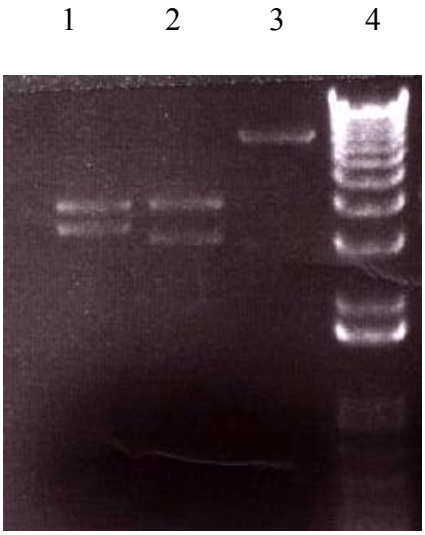

Figure 2.1: The agrose gel indicating the fragments generated by restriction enzyme digestion**.** Lane 1; Single digest, Plasmid digested by *Bam*HI indicates two fragments about 3.7 kb and 3.8 kb in size, *Bam*HI has two restriction sites in the vector as well as the insert. Lane 2; Double digest, Plasmid digested with *Bam*HI and *Sal*I, Both the *Bam*HI and *Sal*I have restriction sites in the vector are very close to each other, the restriction sites differ by 276 bp, hence there is a shift in the band that can be observed on the gel. The released fragment is very small in size so couldn't be detected on the gel. Lane 3; Single digest, Plasmid digested with *Sal*I*,* the plasmid is7.6 kb in size. Lane 4; This lane shows the 1 kb DNA Marker (Promega, USA).

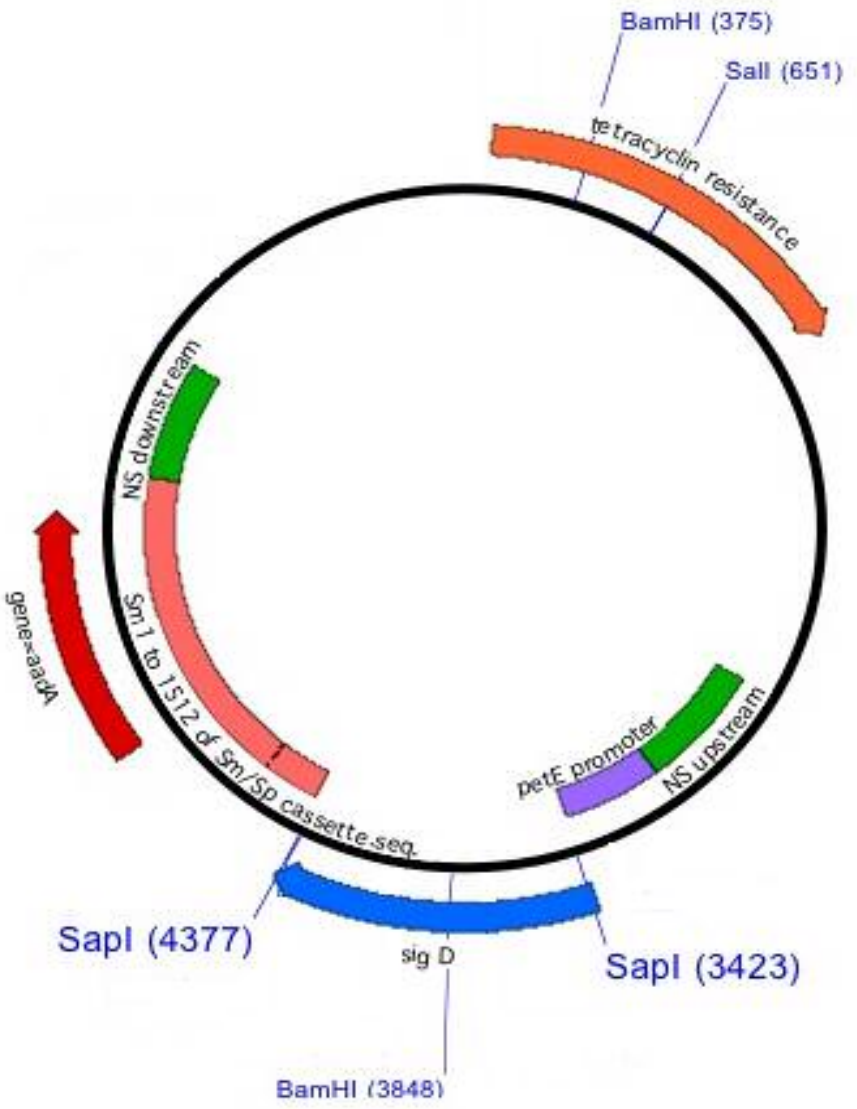

Figure 2.2: The plasmid map of pPETE-sigD: Spec/Ter-low copy pBR328 derivative. The plasmid is 7.6kb in length and the restriction sites that were used for analysis were *Bam*HI and *Sal*I. The map shows the *Bam*HI having two restriction sites each in the vector as well as the insert and *Sal*I having only one restriction site in the vector alone. The flanking sequences of the *sigD* gene contain the *Sap*I restriction sites.

Transformation of pPETE*:sigD* into *Synechocystis* sp. PCC6803

The *Synechocystis* sp.PCC6803 WT cells were grown in 100 ml BG-11 (cyanobacterial bacteria growth media), when the culture reached the mid-log phase, it was used for transformation. Before the transformation was performed the cyanobacterial culture was plated on LB agar plates and incubated overnight at 37°C to check for bacterial contamination. Maintaining sterile conditions the cells were transferred into 250 ml centrifuge bottles and pelleted by centrifugation at 6000 X g at 25°C for ten minutes. The pellet was resuspended in 1 ml BG-11 by vigorous shaking under light for ten minutes. About 300 µl of the culture was transferred into a fresh 14 ml BD Falcon tube and10 µl of plasmid DNA(1 µg/µl) was added, the contents were incubated at 30°C by constant shaking at 250 rpm in the presence of light for five hours. The contents were then transferred into 125 ml flasks and kept shaking under the same conditions overnight. After 24 hours the cells were plated on BG-11 agar plates with 5 µg/ml spectinomycin. After approximately two weeks the colonies were picked and streaked on BG-11 agar plates containing higher concentration of antibiotics (spectinomycin 20 µg/ml). The cells were propagated on BG-11 agar plates with spectinomycin 20 µg /ml for two months for segregation of mutant chromosome. *Synechocystis* sp. PCC6803 is shown to undergo homologous double crossover. The target gene *sigD* with the promoter and the spectinomycin cassette is transformed in to previously defined neutral site in *Synechocytsis* sp. PCC 6803 chromosome by means of double recombination (Williams, 1988).

Growth conditions of *Synechocystis* sp.PCC6803 for high light treatment

Synechocystis sp. PCC6803 cells were grown on autotrophic BG-11 agar plate media with for about two weeks at 30ºC under fluorescent white light. A loop-full containing several colonies was inoculated into 100ml of BG-11 liquid media pH 8.0 into 250 ml Erlenmeyer flask fitted with cotton plug. The culture was grown under the same light conditions while shaking at 200-250 rpm until it reached mid- to late- log phase (OD_{750} reaches between 0.5-1). The mutants were also grown similarly under an antibiotic selection. About 4-5 ml of starter culture was used to inoculate into 300 ml of BG-11 liquid media buffered at pH 8.0 in flat Bellco tissue culture flasks. These bottles were fitted with a bubbling apparatus and aerated with air enriched with 3% CO_2 (v/v). These culture bottles were placed in water tanks with continuous circulation of water to maintain the temperature of the water bath at 30°C. *Synechocystis* sp.PCC6803 cultures were initially illuminated with shaded fluorescent white light (photon flux density = 20 μmoles photons m^{-2} s^{-1}) for about two weeks. This was termed as the lowlight (LL) condition.

Care was taken to make sure that cells were only light limited and not carbon limited. Cell densities were observed regularly and the cultures were diluted with 400-500 ml of BG-11 liquid media when it reached an optical density at 750mn (OD_{750}) ~ 0.8. Cultures were maintained at OD_{750} between 0.4 and 0.8 by periodic dilution. After growing the cells for at least two weeks under these conditions, cells were harvested for RNA isolation at an OD_{750} of 0.5. The light intensity was then increased by using flood lights (photon flux density = 500 μmoles photons m^{-2} s^{-1}) .This was termed as the highlight treatment (HL). The cells were then subjected to these high light treatments for

15 minutes, 30 minutes, 60 minutes and 180 minutes time interval and harvested at each time point.

High light treatment experiments were also performed using cells grown in air without CO_2 enrichment. The difference in the growth set up was that the starter culture was inoculated into 300 ml of BG-11 liquid media buffered at pH 7.5, bubbled with air [0.03% CO_2 (v/v)] instead of air enriched with 3% CO_2 (v/v). The WT cells were grown under the same light conditions as described earlier. The cells were then subjected to the same high light treatments as described earlier for 15 minutes, 30 minutes and harvested at each time point.

Growth conditions of *Synechocystis* sp. PCC6803 in thiol stress

The *Synechocystis* sp. PCC 6803 cells were grown under the same conditions as described earlier. Instead of HL, the cells were treated with 5 mM dithiothreitol (DTT) and the cultures were incubated under the same low light conditions for 15 minutes. A small portion of the culture was harvested for RNA isolation this was used as the 'reference' and the rest were treated with DTT and harvested again after 15 minutes.

RNA isolation

All the reagents were prepared the previous day, the rotor was pre chilled at 4ºC, autoclaved tubes and tips were used. All the solutions were prepared using diethylpyrocarbonate (DEPC) treated water and all reagents were autoclaved prior to use. Two hundred milliliters of *Synechocystis* sp.PCC6803 cultures at OD_{750} of 0.5-0.7 was transferred into 250 ml centrifuge bottles (phenol-resistant) prefilled with 20 ml of 20 X stop solution (100 ml of saturated phenol, 96 ml of 0.5 M EDTA pH 8.0, and 4ml of β-

mercaptoethanol). Cells were harvested by centrifugation at 4ºC, 8000 X g for ten minutes. Supernatant was discarded and the pellets were thoroughly drained and kept in cold by placement on ice. Pellets were then resuspended in 600 µl of cell suspension buffer (0.3 M sucrose, 10 mM sodium acetate pH 4.5). Resuspended cells were transferred into a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 10,000 X g for one minute at room temperature. Supernatant was discarded and pellets were flash frozen in liquid nitrogen. The protocol can be stopped at this point and continued later.

Cells were thawed on ice, this rapid freezing and later thawing facilitates the breakage of the cells. This was followed by the addition of 38 µl 0.5 M EDTA and 320 µl of ice cold cell suspension buffer to resuspend the cells again. Further 340 µl of sodium acetate pH 4.5 (this was prepared mixing 1 part of 1 M sodium acetate pH 4.5 and 8 parts of DEPC treated water) and 38 µl of 20% SDS were added, mixed well and incubated at 65ºC for ten minues (The contents were mixed thoroughly during incubation time by inverting the tubes). This was followed by the addition of 700 µl hot acidic phenol (Ambion, USA) (65ºC), and incubated at 65ºC for five minutes. The contents were mixed thoroughly during the incubation time by inverting the tubes. Tubes were immediately cooled by placing at -80ºC for 45 seconds and aqueous phase was separated by centrifugation at 10,000 X g for five minutes at room temperature. The aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube and extracted with 700 µl of hot acidic phenol one more time as described earlier. The aqueous phase was again extracted using 700 µl of 1:1 saturated phenol:chloroform solution.

The nucleic acids were differentially precipitated using .25 volumes of 10 M lithium chloride and 2.5 volumes of absolute ethanol, incubated for 90 minutes at -20ºC.

Contents were centrifuged and the pellet was washed twice with 70% ethanol and air dried. The dried pellet was dissolved in 50 µl of DEPC treated water. RNA was stored at -80ºC for later use. About 1 µl of RNA was used for quantification using NanoDrop® ND-1000 Spectrophotometer (NanoDrop technologies, USA). The quality of RNA was also analyzed using 0.8% agrose gel electrophoresis. RNA isolated using phenol chloroform methodology was found to contain traces of contaminating genomic DNA. The residual DNA was hydrolyzed and the RNA was further purified and concentrated using Ambion DNA free™ kit followed by ethanol precipitation.

RNA cleanup using "Ambion DNA free™ kit"

One microlitre of rDNase I enzyme (2 Units) was used to remove genomic DNA contamination in 10 µg of RNA in a 50 µl reaction. About 0.1 volumes of 10 X DNase I buffer and 1 µl rDNase I was added to 10 µg of RNA in a 50 µl reaction. The contents were gently mixed and incubated at 37ºC for 30 minutes. DNase Inactivation Reagent was completely resuspened by flicking or vortexing before dispensing 0.1 volumes of the reagent into the reaction mixture. The contents of tube were mixed well and incubated for two minutes at room temperature. During incubation period the contents were mixed two to three times to redisperse DNase Inactivation Reagent. The reaction mixture was centrifuged at 10,000 X g for one-and-half minute. The DNA-free RNA was contained in the supernatant was transferred into a fresh tube.

Ethanol precipitation of DNA-free RNA

The DNA-free RNA was further concentrated using ethanol precipitation. The total volume of RNA available was recorded and 0.5 volumes of 7.5 M of ammonium

acetate and 2.5 volumes of cold absolute alcohol were added. Incubated at -20ºC overnight and centrifuged at 14,000 X g for ten minutes. Supernatant was discarded and the pellet was vacuum dried for ten minutes. Pellet was dissolved in 20 µl of DEPC treated water and stored at -80ºC. Two microlitre of RNA was run on 0.8% agrose gel to check for traces of genomic DNA. RNA was quantified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop technologies, USA).

Preparation of fluorescently labeled cDNA (complementry DNA)

The cDNA was labeled using fluorescent dyes by means of a two step indirect labeling technique. The first step involved cDNA being synthesized from target RNA by using a reverse transcriptase and deoxynucleotides containing an aminoally-modified deoxyuridine triphosphate (aadUTP). Resulting cDNA contained amino moieties that were involved in chemical coupling with fluorescent dyes namely Alexa 647 or Alexa 555 (Invitrogen, USA). The cDNA was synthesized from control and experimental RNA samples separately. The cDNA was synthesized from 7 µg of total RNA using 8 µg of Genisphere array 900 MPX random primers, total volume was brought up to 25 µl using DEPC-treated water (a proprietary mixture found to give the highest yields) . The reaction was carried out in thin walled PCR tubes (Eppendorf, USA) using thermocycler. In order to facilitate annealing of the primers and the subsequent binding to the template, temperature was set at 80°C for ten minutes in a thermocycler. Followed by chilling on ice for two minutes and then added 25 µl of 2 X reverse transcriptase master mix to each of the tubes.

Two times reverse transcription (RT) master mix was prepared using 17.5 µl of DEPC-treated water, 12.5 µl of 0.1 M DTT, 25 µl of 5 X First Strand Buffer (FSB), 2.5

µl of 50 X aadNTP (amino allyl dNTP) mix that consists of 25 mM dATP (Invitrogen, USA), 25 mM dCTP (Invitrogen, USA) , 25 mM dGTP (Invitrogen, USA), 8.75 mM dTTP (Invitrogen, USA) and 16.25 mM aad UTP (Sigma Cat No.-A-5660, USA) and 5µl of Superscript II (Invitrogen, USA). The tubes were incubated at 42°C in a thermocycler for three hours.

RNA that acted as the cDNA template was hydrolyzed by adding 2.5 µl of 10M sodium hydroxide prepared freshly and 5 µl of 50 mM EDTA. The reaction was carried out at 65°C for 20 minutes and the alkali was neutralized by the addition of 5 µl of 5 M acetic acid. The amino allyl modified cDNA was purified using Millipore Microcon YM 30 filtration devices. The clean up columns were prewashed with 100 µl of sterile deionized nuclease free water and centrifuged at 14,000 X g for five minutes. Each sample of aadUTP tagged cDNA populations was transferred to the columns with 450 µl of sterile deionized nuclease free water. The columns were centrifuged at 14,000 X g for 20 minutes. Columns were washed again three more times using 450 µl of sterile deionized nuclease free water. After the last wash 10 µl of sterile deionized nuclease free water was added to the column membrane, incubated at room temperature for five minutes. The column was then transferred the column into a fresh Eppendrof tube and centrifuged at 14,000 X g for two minutes. The amino allyl modified cDNA thus synthesized was quantified using NanoDrop® ND-1000 Spectrophotometer. Only the reactions that yielded 5 µg or greater cDNA were further processed.

The volume of amino allyl modified cDNA or the target cDNA was reduced to 6 µl using a Speed Vac and the target cDNA was diluted with 2µl of 0.5 M sodium carbonate pH 9.0 (prepared fresh from a stock solution of 1M sodium carbonate). Prior to

the coupling reaction, the fluorescent dyes (Alexa fluor 647 and Alexa fluor 555) were separately dissolved in 2 µl of high quality dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). To ensure the dye was completely dissolved the vials were vortexed for ten seconds. Once the dyes were dissolved, the reaction was performed immediately. To initiate the coupling reaction, 8 µl of amine modified cDNA was added to the freshly prepared fluorescent dyes. The coupling reaction was carried out in the dark for an hour at room temperature. Reaction was quenched by the addition of 4.5 µl of freshly prepared 4 M hydroxylamine. Quenching reaction was carried out in the dark at room temperature for fifteen minutes. The Alexa Fluor 647 or Alexa Fluor 555 dyes coupled with cDNA were combined and the unincorporated dye was removed by using QIAquick PCR Purification kit (Qiagen, USA). Both the samples were mixed followed by the addition of 35 µl sterile deionized nuclease free water, 35 µl of 100 mM sodium acetate pH 5.2 and 500 µl buffer PB. The entire contents were applied to the column and centrifuged at 14,000 X g for one minute. The column was washed four times with 600 µl buffer PE and centrifuged each time at 14,000 X g for one minute. The empty column was centrifuged again to remove any residual buffer. Column was transferred to a fresh tube and 15 µl of sterile deionized nuclease free water was added. The column was kept at room temperature for five minutes and then spun at 14,000 x g for a min. The elution step was repeated with additional 15 µl sterile deionized water.

The stock solution of 2 X formamide-based hybridization (50% formamide, 10 X SSC, 0.2% SDS) was completely dissolved by heating at 65°C for at least ten minutes on a heating block. Since 25 X 60 LifterSlips (Erie Scientific, USA) were used 5 µl of sterile deionized nuclease free water and 35 µl of completely dissolved 2 X formamide-based

hybridization buffer was added to the 30 µl of labeled cDNA to achieve a final volume of 70 µl for hybridization (Postier et al., 2003).

DNA microarray slide preparation

The slide is prepared for pre-hybridization prior to the coupling reaction. The printed slides were baked and stored in a dessicator for long term storage purposes. The slide was initially subjected to UV crosslink at 150 mJ/cm^2 (1500 X 100 uJ/cm^2 energy setting) to ensure the probes were strongly bound to the glass slide. The slide was rinsed with 0.1% SDS at room temperature for two minutes to wash off excess unbound DNA. Slide was again rinsed in sterile deionized water for two minutes at room temperature. Probes were denatured by boiling the slide in a breaker of sterile deionized water for three minutes. The slide was then immediately transferred into 95% cold ethanol (-20°C) and spun dried using Telechem microscope slide drier. The slide was then incubated at 42°C for one-six hours in the pre-hybridization solution (25ml of 25% Formamide, 5 X SSC, 0.1% SDS and 1% BSA)

Hybridization

Slide was rinsed twice with sterile deionized water spun dried. The slide was prewarmed by placing it in a Corning hybridization chamber along with 20 X 60 LifterSlips. The formamide based hybridization mix was incubated at 80-99°C for ten minutes to denature the fluorescently labeled cDNA. The contents were cooled briefly by centrifugation for 30 seconds and kept at 42°C. The prewarmed hybridization chamber was disassembled and the hybrization mix containing the labeled target was applied at the edge of the coverslip on the surface of the prewarmed slide that allowed

the labeled cDNA to be carried over the entire printed area by capillary diffusion. About 15 µl of 3 X SSC was added to the buffer wells on each end and the hybridization chamber was reassembled. The chamber was returned to the static incubator and incubated for about sixteen hours at 42°C (Postier et al., 2003).

Post -hybridization stringency washes

The hybridization chamber was disassembled and the slide was placed in 2 X SSC, 0.1% SDS for 5 min at 42°C. The coverslip was allowed to fall off on its own without application of any force. The slide was again rinsed in 0.1 X SSC, 0.1% SDS for ten minutes at room temperature and in 0.1 X SSC for one minute at room temperature (this step was repeated four times). Finally slide was washed in sterile deionized water, dried by spinning and scanned (Postier et al., 2003).

Scanning/ Signal detection

The slide was scanned using GenePix 4000B microarray scanner (Axon Instruments Inc., Union City, CA). The scanner was interfaced with a computer that enabled the use of GenePix Pro (GPP, version 4.1) software. The scanned images were saved as TIFF files. The spot finding was done by loading the GAL file which is a text file containing gene descriptions and the coordinates on the array (defined as blocks, rows, name and id).

Global gene expression analysis

The GenePix pro generates data that was used for downstream analysis and processing. The resultant files are called as GPR (GenePix Pro) files and contains information about signal intensities $\log_2$ ratios and the basic statistical information for

both wavelengths. These GPR files are used for analysis by using GenePix Auto Processor 32 (GPAP) software available at the Microarray core facility website (http://darwin.biochem.okstate.edu/gpap, Oklahoma State University) (Weng, 2005). This software is available online and could be accessed easily. The GPAP aids in the preprocessing that includes correction, filtering and normalization of raw microarray data. GPAP uses R statistical software with Biconductor and LIMMA packages. The output after this preprocessing is in the form of reports and diagnostic plots. These plots enable to evaluate data quality and the outcome of preprocessing. This software also does a number of data processing operations such as signal filtering, background correction and signal normalization. The following user defined parameters were set before the data was processed (Ayoubi, 2005)

Baseline value: The baseline values of the fluorescence intensity were set to 200 or above to filter out low intensity spots. For any spot with a baseline value of 200 or above in both the channels was taken into consideration. If the spot intensity was less than 200 then it was discarded because it was unreliable. Values between 10 and 100 are typical of background fluorescence whereas strong signals have values above 200

Normalization: There are two kinds of normalization involved here.

1. Normalization within an array – This was done using Local Lowess –Pin by pin intensity dependent normalization. This was non-linear normalization.
2. Normalization between two arrays- This was done using Quantile normalization.

All these parameters aid in identifying the valid features and there by minimizing noise and poor quality features. Normalization is essential for microarray data because that helps to amend any changes caused due to non-biological artifacts such as printing,

labeling hybridization and scanning. Normalization cannot correct biological differences between the samples.

GPAP analysis generates the following reports:

Gene Summary (averaged) - This report contains normalized average Log_2 ratios (M), Average intensity or total intensity A was calculated as $1/2(\log_2$ (Alexa647*Alexa555)) for each gene with in the array and between the replicate slides. This report also includes standard deviation, coefficient of variation, number of replicates that were included in calculating the averages, number of outliers that were not included in calculation of the averages for each gene (Ayoubi, 2005).

B-statistics Ranking- GPAP ranks the genes by B-statistic based on a user defined cut-off. The B-statistics ranking report lists all genes contains all the invalid features with flags <0, features that did not pass the user defined baseline values, threshold values or saturated in both channels were included in the calculation of normalized averaged $\log_2$ ratio but were penalized by assigning a low weight of '0.1'. This report also calculates p-value and t-statistic for the average $\log_2$ ratio for each gene. B-statistic is much more robust statistical analysis to avoid false positives or false negatives (Ayoubi, 2005).

CHAPTER IV

GLOBAL GENE EXPRESSION PROFILING OF *SYNECHOCYSTIS* sp.PCC6803 IN HIGH LIGHT CONDITIONS

I. Introduction

Acclimation to high light both short term and long term processes in *Synechocystis* sp. PCC6803 have been extensively studied by various research groups (Hihara et al., 2001; Tu et al., 2004). Physiological responses in low light, high light and also light under different wavelengths have been investigated (El Bissati and Kirilovsky, 2001; Huang et al., 2002). My objective was to understand the regulation of a set of genes involved in the repair of PSII. D1 is the core of PSII and this protein undergoes rapid turn-over under light. In *Synechocytsis* sp. PCC6803*,* D1 protein undergoes rapid turnover and this turnover depends on the balance between rates of photodamage and repair (Allakhverdiev et al., 2005). Studies have shown that multiple genes with varying degrees of certainty play role in D1 turnover, these include *psbA2 (slr1311)*, *psbA3 (sll1867)*, *sigD (sll2012), ftsH (slr0228)* (Hihara et al., 2001; Imamura et al., 2003b; Nixon et al., 2005). These and other genes may be coordinately expressed under conditions that result in damage to PSII.

Earlier microarray work based upon high light, redox, low inorganic carbon stress and all conditions leading to PSII damage, have revealed that these genes were induced to high levels (Hihara et al., 2001; Hihara et al., 2003; Wang et al., 2004). Therefore these genes may be a part of a hypothetical 'PSII repair regulon'. The foundation for my thesis was to understand the possible role of *sigD* in the PSII repair, since SigD expression correlate with the expression of genes in this hypothesized regulon.

When there is an imbalance between the rates, i.e. if the rate of photodamage exceeds repair, PSII succumbs to photoinhibition. As noted several studies have shown that the expression of *sigD* increases under conditions that result in high rates of D1 repair (Imamura et al., 2003a; Imamura et al., 2003b; Wang et al., 2004). This correlation, while suggestive is not sufficient to establish a more mechanistic connection. More insight to the mechanism have come from studies on *sigD* showing promoter specificity of *sigD* and more clearly defining its high light responsiveness (Imamura et al., 2003b). Earlier experimental conditions to understand the function of SigD indicated that the cells were subjected to darkness for few hours and then exposed to high light (Imamura et al., 2003a). I decided to modulate the experimental design so instead of growing the cells in darkness and then exposing to high light. The cultures were grown in low light and then subjected to high light stress. This is more generally used approach since it reflects many fluctuations of light intensity in nature. In this chapter, I will also describe experiments better defining the high light response, the role of *sigD*, and how the high light response appears to be integrated with other stress responses.

I will also be emphasizing the discrepancies in the earlier published microarray data (Hihara et al., 2001), that exhibited a small subset of high affinity carbon

transporters being induced that wasn't observed in the HL experiments performed. But these transporters were up-regulated when the cells were grown in moderate inorganic carbon (grown in air) then exposed to HL. Thereby by performing the HL experiments I was re-investigating the HL response to bring out the disparities and also try to understand the genes regulated by *sigD* in the PSII repair regulon.

II. Results and Discussion

A. Re-investigation of the high light response in *Synechocystis* sp. PCC6803

Importantly, the conditions of inorganic carbon availability were maintained very high in this first set of the high light experiments. Note, that in one set of experiments, described in section C of this chapter, the availability of carbon was maintained lower, but not limiting condition prior to high light treatment. In all cases, RNA isolated at low light was considered as the "reference sample" and RNA extracted at high light at different sampling time points was considered as the "treatment sample". The RNA extraction and growth conditions are discussed in materials and methods. The microarray hybridizations for WT stain and the dyes used are summarized in table 3.1.

Strains	Hybridizations	Dyes used
Synechocystis Wild Type	WTLL-WTHL15min	Alexa555 vs. Alexa647
	WTLL-WTHL15min	Alexa647 vs. Alexa555
	WTLL-WTHL30min	Alexa555 vs. Alexa647
	WTLL-WTHL30min	Alexa647 vs. Alexa555
	WTLL-WTHL60min	Alexa555 vs. Alexa647
	WTLL-WTHL60min	Alexa647 vs. Alexa555
	WTLL-WTHL180min	Alexa555 vs. Alexa647
	WTLL-WTHL180min	Alexa647 vs. Alexa555

Table 3.1: The list of microarray hybridizations for WT. The hybridizations were done twice using biological replicates. The microarray hybridizations for the different time points of high light exposure is summarized in this table.

Hybridizations were done in duplicates with dye swaps. The replicates were un-pooled independent biological replicates (two WT *Synechocystis* sp.PCC6803 cultures were grown identically under the same conditions) and each array had three spots for a single gene. Hence there were six replicates for a single gene for each time point. The $\log_2$ ratios were calculated as $\log_2$ (Treatment/ Reference) or $\log_2$ (Mutant/WT) depending on the hybridization. The results of the high light treatments at different time points are discussed in detail in the following sections.

Microarray analysis of high light treatment in the WT

Correlation coefficient between the biological replicates after preprocessing is illustrated in table 3.2. The correlation coefficient is important statistic that represents the similarity between the biological replicates. Perfect correlation is '1' and no correlation is '0', correlation coefficient demonstrates the reproducibility of microarray data between different biological replicates.

Hybridization	Correlation between the biological replicates
WTLL-WTHL15min	0.7758
WTLL-WTHL30min	0.7863
WTLL-WTHL60min	0.9077
WTLL-WTHL180min	0.8189

Table 3.2: The correlation coefficient between the biological replicates in the WT in the HL treatment for different sampling time points.

Higher the correlation the greater is the similarity between the biological replicates. The threshold for significance was based on p-value < 0.05 (false discovery rate) for at least one time point.

There were 3168 ORFs of *Synechocystis* sp. PCC6803 spotted on the arrays including three replicate spots for a gene and there were two biological replicates for each

time point. Based on the threshold values in WTLL- WTHL experiment, out of 3168 genes, only 575 ORFs exhibited at least two- fold change or greater in at least one of the four sampling time points.

Increased transcript abundance or decreased transcript abundance for a particular transcript is determined by its synthesis or degradation, microarray analysis is incapable of distinguishing between them, hence for simplicity, I have used the terms 'up-regulated' for transcripts that have been induced or are more abundant in the treatment compared to the reference and 'down-regulated' for the transcripts that are less abundant in the treatment as compared to the reference.

Hierarchical clustering analysis

Cluster analysis was done using the Genesis –Gene Expression Similarity Investigation Suite (Sturn, 2000), the program clusters the genes based on the patterns of expression. Genesis is a flexible program that assists in analyzing multiple genes or cluster of genes in terms of their similarity, functions, behaviors, or interactions (Ayoubi, 2005). If multiple genes are a part of an operon, they are grouped together because they tend to be co-regulated barring significant transcriptional attenuation or multiple transcriptional start sites. Such gene therefore may exhibit similar pattern of expression and tend to be clustered together by this analysis. There are various types of cluster analysis that can be performed such as hierarchical clustering, K-means non hierarchical clustering, and Self organizing maps. The cluster analysis is tertiary level of microarray data analysis, the data obtained after stringent cut-off and normalization is loaded into cluster analysis programs to identify genes that have similar patterns of expression or are co-regulated in the same manner (Ayoubi, 2005).

Initial analysis of the data processing based on the statistical criteria was conducted before hierarchical clustering. In hierarchical clustering, genes were grouped on the basis of their similarities in differential expression changes. For example, a group of genes rapidly induced and maintained at high levels through out the experiment would be clustered together in one group or cluster. Genes that are not significantly up-regulated early in the experiment but gradually increase would fall in another cluster. The algorithm creates a dendrogram organization of genes clustered in this way that makes visual identification of similarly regulated genes easier. Using cluster analysis a hypothesis can be developed about the function of poorly characterized genes on the basis of their kinetic association with known genes (for example, functional cluster VII *hli* genes and *slr1544*, table3.9).

The hierarchical clustering was conducted and then the supervised clustering was done, the genes were classified in to groups based on their function. The WTLL was used as the reference, there were 575 ORFs which consistently displayed expression changes of two fold or greater ($\log_2 < -1$ or $\log_2 > 1$) for at least one of the four sampling time points (15minutes, 30minutes, 60minutes, 180minutes). Based on their expression pattern and their function, the ORFs were classified into the following clusters. The synchronized expression pattern (up-regulation or down-regulation) of genes actually clustered together on the chromosome could be associated with operon structure.

Based on the supervised clustering the genes were grouped in the following cluster as described below

1. Functional Cluster -I (Chaperonins) - This category included heat shock proteins and protein folding molecular chaperones.

2. Functional Cluster-II (Photosystem I genes) - This group includes genes that encode for the different subunits of Photosystem I
3. Functional Cluster-III (Phycobilisome related genes) - The group includes the genes coding for light harvesting antenna complexes
4. Functional Cluster-IV (Photosytem II genes) - This category consists of the genes encoding Photosystem II subunits including the core and the peripheral subunits
5. Functional Cluster-V (Chlorophyll and heme biosynthesis genes) - This group consists of genes that encode for different enzymes involved in chlorophyll and heme biosynthesis.
6. Functional Cluster-VI (Ribosomal related genes) - This cluster includes genes transcribing the ribosome 50S subunits and 30S subunits.
7. Functional Cluster-VII (High light inducible genes) - This cluster includes the genes that code for high light inducible polypeptides and also few hypothetical proteins.
8. Functional Cluster-VIII (PSII repair genes) - This cluster is the genes that are hypothesized to be responsible for the PSII repair. The cluster includes *psbA2*, *psbA3*, *ftsH(slr0228), ftsH(slr1604)*, *sigD*, and *groEL-2*.

Functional Cluster -I (Chaperonins)

This category includes genes such as *dnaJ, dnaK1, dnaK2, groEL, hspA, groEL-2, htpG, ycf39* (chaperon-like protein for quinone binding in photosystem II), The heat shock proteins are recruited for folding proteins and refolding of old proteins. As depicted in the table 3.3, the 60kD molecular chaperonins *groEL* exhibited almost four-fold increase at 15 minutes of high light exposure and reduced to almost two-fold after 30 minutes, and retained the same for three hours. On the other hand *groEL-2* is highly induced to almost twelve-fold in the initial 15 minutes of exposure to light (Table 3.3). The same scenario was observed with heat shock proteins such as *hspA* (16.6kD small heat shock protein) and *htpG* both were highly up-regulated at 15 minutes but reduced to almost two-fold after three hours. The up-regulation exhibited by other heat shock proteins in this group such as *dnaJ*, *dnaK2, dnaK1* were also high at 15 minutes. These findings are consistent with the fact that protein folding is affected by heat and the same holds for HL. The proteins may undergo structural or conformational change. Hence the chaperones are engaged to repair the damage caused by misfolding of proteins. The relative fold changes for Cluster –I are represented in table 3.3 and the graph (figure 3.1) represents the overview of all genes belonging to this cluster

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
slr0093	***dnaJ***	**5.3**	**1.0**	**1.1**	**1.2**
sll0897	***dnaJ***	**3.2**	**1.2**	**1.1**	**1.3**
sll0170	***dnaK2***	**7.2**	**1.4**	**1.1**	**1.6**
sll0058	***dnaK1***	**12**	**1.5**	**1.0**	**1.1**
slr0399	***ycf39***	**4.9**	**2.4**	**2.9**	**2.5**
slr2076	***groEL***	**3.9**	**2.8**	**4.0**	**4.6**
sll1514	***hspA***	**25.3**	**2.1**	**1.9**	**4**
sll0430	***htpG***	**22.5**	**2.5**	**2.8**	**2.8**
sll0416	***groEL-2***	**12.6**	**4.4**	**8.7**	**6.6**

Table 3.3: Relative fold change for Cluster -I. Cluster-I consists of various heat shock proteins and molecular chaperonins. There was high induction at 15 minutes followed by a decrease in fold induction over time. Except for *groEL* that was induced through out the high light treatment transcripts encoding for all other molecular chaperonins were decreased after prolonged exposure to high light.

Figure 3.1 represents the accumulation of transcripts coding for heat shock proteins and molecular chaperonins during the high light treatment. The transcripts belonging to this cluster demonstrated a high induction at 15 minutes and then there was a substantial decrease in the level of induction after prolonged exposure to light.

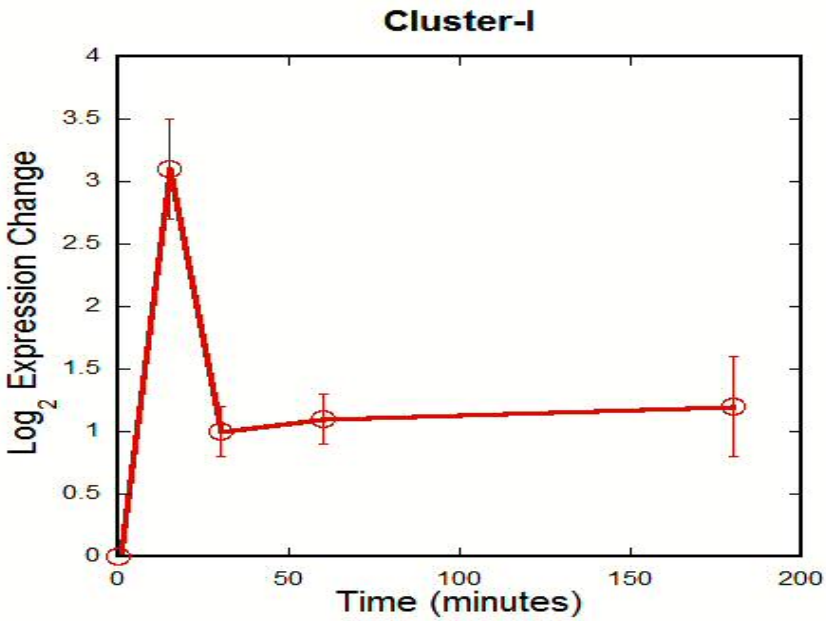

Figure 3.1: Overview for Cluster-I. The X axis depicts the time points after exposure to HL, and the Y axis shows the average $\log_2$ expression ratios for cluster-I. There was an initial induction of almost eight-fold at 15 minutes.

Functional Cluster –II (Photosystem I genes)

The genes belonging to this cluster encode for various subunits of PSI. All the transcripts in this category displayed marked down-regulation almost five to ten-fold within 60 minutes of transition from low light to high light. This is a common characteristic observed in cyanobacteria when exposed to high light. In high light, there is a high influx of photons, this leads to imbalance between energy absorbed and energy dissipated. This imbalance between energy supplied and energy consumed leads to photodamage. Therefore as a protective measure against photodamage, decrease of PSI content is the typical response by cyanobacteria during the acclimation to HL. The genes included in this cluster are *psaA*, *psaB* that encodes P700 apoprotein subunit Ia and P700 apoprotein subunit Ib, respectively. The genes *psaC*, *psaD*, *psaE*, *psaI, psaJ, psaK*, *psaL* and *psaM* encode for photosystem I subunits VII, II, IV, VIII, IX, X, XI and XII

respectively, *psaF* encodes for reaction center subunit III (plastocyanin docking protein). The fold changes for the transcripts are displayed in the table 3.4. The over view of genes in this cluster view is also illustrated in the figure 3.2

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
slr1834	***psaA***	**-2.4**	**-6.9**	**-29.6**	**-24.8**
slr1835	***psaB***	**-4.2**	**-5.6**	**-26**	**-21.8**
ssl0563	***psaC***	**-4.2**	**-8.9**	**-17.3**	**-18.2**
slr0737	***psaD***	**-5.3**	**-8.9**	**-18.6**	**-16.9**
ssr2831	***psaE***	**-7.1**	**-8.7**	**-12.4**	**-11.4**
sll0819	***psaF***	**-8.2**	**-17**	**-18**	**-14.5**
smr0004	***psaI***	**-2.8**	**-1.9**	**-29**	**-13.8**
sml0008	***psaJ***	**-8.6**	**-17.8**	**-20.4**	**-18**
ssr0390	***psaK***	**-5**	**-12.9**	**-26.2**	**-17.4**
slr1655	***psaL***	**-10.1**	**-13.8**	**-19.8**	**-21**
smr0005	***psaM***	**-4.3**	**-3.9**	**-11**	**-11.2**

Table 3.4: Relative fold change for Cluster-II. The genes that encode for the different subunits of photosystem I belong to Cluster-II. There was a marked down-regulation in transcripts abundance belonging to this cluster as a result of the transition from low light to high light and the down-regulation becomes prominent over time. The fold change is indicated with a negative sign to emphasize that these genes were down-regulated.

The figure 3.2 depicts change in the expression ratios over time. The transcripts exhibited massive down-regulation during the transition from low light to high light. The transcripts were almost five to twenty-fold down-regulated during the high light treatment

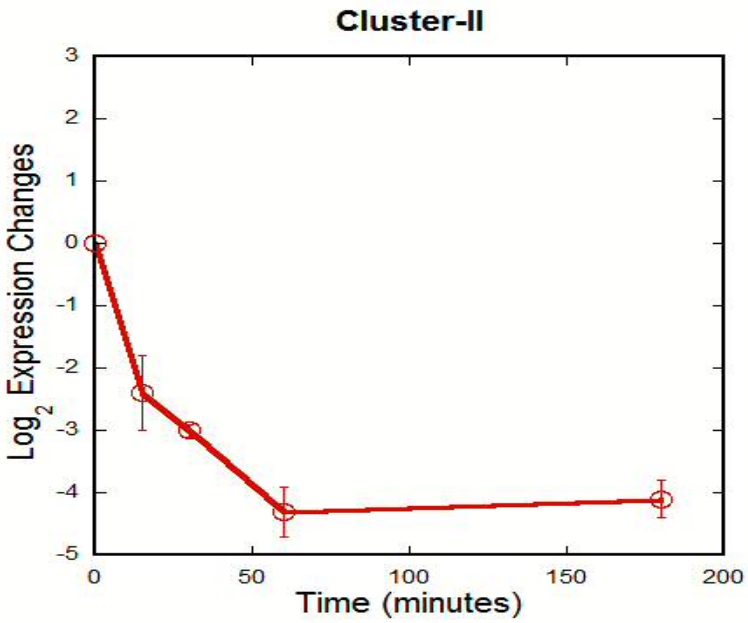

Figure 3.2: Overview of Cluster-II. The X axis depicts the different time points and the Y axis depicts the average log_2 expression ratios for the genes belonging to Cluster-II. There was a massive down shift observed in the transcript levels of the genes belonging to this cluster and it persisted during the entire HL treatment.

Functional Cluster –III (Photosystem II genes)

The genes that belong to this cluster encode proteins that comprise the multi subunit PSII complex. These include the extrinsic, water-soluble membrane proteins that form the exterior shield for the manganese complex, cytochrome c550 encoded by *psbV*, manganese –stabilizing polypeptide encoded by *psbO* and *psbU*. The cluster also includes numerous large and small intrinsic membrane proteins such as photosystem II core light harvesting protein CP47 encoded by *psbB*. Most genes for PSII proteins were found to be down-regulated. However, there is an important exception to this general trend. The core subunits were up-regulated and these include *psbA1*, *psbA2*, *psbA3* genes that encode for the D1 protein. Additionally, the transcript levels of *psbD* and *psbD2* genes that encode for photosystem II reaction center D2 protein were also up-regulated. The transcript abundance for these genes were up-regulated as D1 but to a lesser extent as D1 undergoes

rapid turnover under high light. The *psbC* gene that encodes for CP43 and *psbB* were down-regulated. Therefore except for the core proteins D1 and D2, all other subunits of PSII were down-regulated. This was consistent with the down-regulation of PSI subunits in HL. The repression observed in PSI transcripts was more pronounced than in PSII transcripts. The relative fold change in the core and the peripheral subunits of PSII are provided in the table 3.5. The over view of the cluster for the PSII core subunits (D1 and D2) and the peripheral subunits that includes all the other proteins subunits that make up PSII are displayed in figure 3.3.

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
slr1181	***psbA1***	**3.0**	**2.1**	**2.8**	**3.2**
slr1311	***psbA2***	**1.7**	**1.8**	**3.7**	**3.8**
sll1867	***psbA3***	**2.5**	**3.9**	**6.8**	**6.4**
sll0849	***psbD***	**1.2**	**1.7**	**2**	**1.9**
slr0927	***psbD2***	**1.3**	**1.8**	**3.4**	**2.9**
slr0906	***psbB***	**-2.7**	**-2**	**-2.2**	**-1.8**
ssr3451	***psbE***	**-1.8**	**-2.1**	**-3**	**-3**
smr0006	***psbF***	**-2.0**	**-2.4**	**-3**	**-2.9**
ssl2598	***psbH***	**-1.9**	**-2.1**	**-2.3**	**-2**
sml0001	***psbI***	**-2.1**	**-1.7**	**-2.5**	**-2.2**
smr0008	***psbJ***	**-1.8**	**-1.4**	**-2.4**	**-2.3**
sml0005	***psbK***	**-1.1**	**-1.6**	**-2.1**	**-2.0**
smr0007	***psbL***	**-2.0**	**-2.0**	**-2.6**	**-2.8**
sll0427	***psbO***	**-3.2**	**-3.3**	**-2.6**	**-2.4**
sll1194	***psbU***	**-2.0**	**-2.0**	**-1.9**	**-2.1**
sll0258	***psbV***	**-1.9**	**-2.3**	**-2.6**	**-4.4**
sll1398	***psbW, psb13, ycf79***	**-1.7**	**-1.8**	**-2.2**	**-1.9**
sml0002	***psbX***	**-1.7**	**-1.0**	**-1.9**	**-2.1**

Table 3.5: Relative fold change for Cluster-III. The genes that encode for the different subunits of photosystem II belong to Cluster-III. The genes that encode the peripheral subunits of PSII were down-regulated where as the core subunits were up-regulated. The negative sign for fold change indicates down-regulation.

Figure 3.3 indicates the change in gene expressions after exposure to HL. Except for *psbA1*, *psbA2*, *psbA3*, *psbD* and *psD2*, all the other gene transcripts forming the PSII complex exhibited down-regulation during the transition from low light to high light.

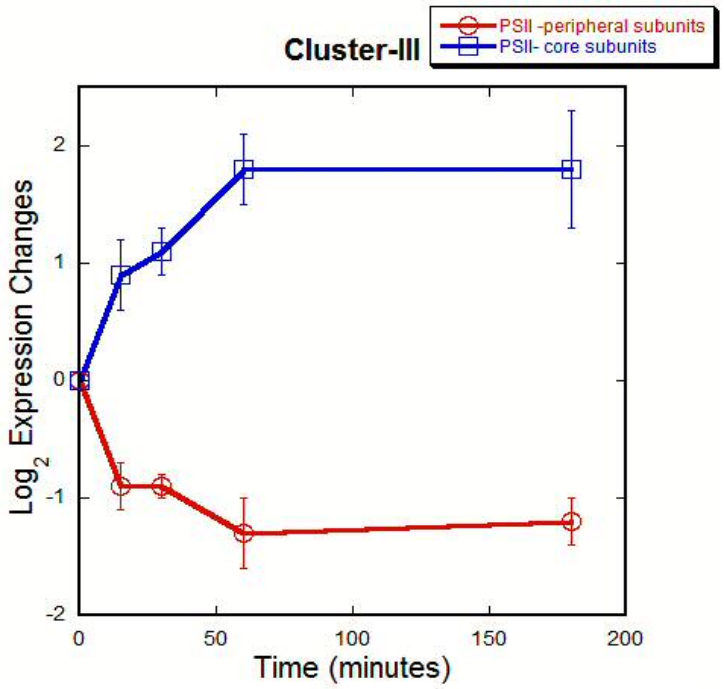

Figure 3.3: Over view of Cluster-III. The *psb* transcripts that form the peripheral subunits of photosystem II were down-regulated in the HL treatment and the *psbA* and the *psbD* were up-regulated in the HL response. The X axis portrays the time points after exposure to HL and Y axis portrays the average $\log_2$ expression ratios for the transcripts.

Functional Cluster –IV (Phycobilisome related genes)

The cluster consists of genes that encode for allophycocyanin and phycocyanin proteins that are involved in light harvesting. In high light these proteins are down-regulated in order to reduce the antenna size. This is to avoid absorption of excess light energy. This is one level of protective mechanism since the excess flux of photons exceeds the amount that can be utilized by photosynthesis mechanism. If the energy that can be utilized is less than the energy absorbed then this excess flux of electrons produced by high rates of photo absorption results in the formation of reactive free radicals. Further more, excess excitation energy not used to drive electron transport can cause the formation of triplet states of chlorophyll that can also sensitize the formation of reactive oxygen species.

The reactive species primarily target PSII causing photoinibition. In order to protect against photodamage in HL, the down sizing of light harvesting complexes (LHC) occurs. The *cpc* transcripts were more predominantly down- regulated than *apc* genes, because the phycocyanin proteins are located at the terminal ends of phycobilisome rods and hence are more sensitive to change in HL intensity. The *cpcBACCD* functions as a polycistronic operon and all the genes contained in this operon such as *cpcA*, *cpcB*, *cpcC1*, *cpcC2* and *cpcD* were down-regulated to the same extent. The requirement to reduce the antenna size could the reason for the up-regulation seen in the transcripts encoding for the phycobilisomes degradation gene *nb1A1*and *nblA2* (Hihara et al., 2001). The transcripts for *nblA1* were up-regulated to 3.8-fold at 15 minutes of HL exposure. The *nblA* is essential for degradation of LHC and down-regulation of PSII activity during

nutrient stress and also HL (Grossman, 1998). The relative fold change in this cluster is represented in table 3.6 and the overview for this cluster is displayed in figure 3.4.

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
slr2067	***apcA***	**-1.1**	**-2.5**	**1.7**	**1.1**
slr1986	***apcB***	**-1.5**	**-4.1**	**-1.0**	**-2.1**
ssr3383	***apcC***	**-5.0**	**-5.0**	**-6.1**	**-7.8**
sll0928	***apcD***	**1.1**	**1.9**	**2.9**	**1.8**
slr0335	***apcE***	**-3.8**	**-3.2**	**-3.7**	**-5.7**
slr1459	***apcF***	**-6.5**	**-7.4**	**-7.7**	**-8.5**
sll1578	***cpcA***	**-5.5**	**-25.6**	**-26.6**	**-47.1**
sll1577	***cpcB***	**-1.3**	**-4.5**	**-2.2**	**-4.4**
sll1580	***cpcC1***	**-5.8**	**-20.4**	**-33.5**	**-43.1**
sll1579	***cpcC2***	**-5.2**	**-19.0**	**-55.4**	**-58.2**
ssl3093	***cpcD***	**-5.8**	**-13.4**	**-27.1**	**-22.0**
slr2051	***cpcG1***	**-7.3**	**-12.0**	**-6.3**	**-9.9**
sll1471	***cpcG2***	**-10.7**	**-23.0**	**-108.7**	**-40.0**
ssl0452	***nblA1***	**3.8**	**1.7**	**2.9**	**2.3**
ssl0453	***nblA2***	**3.4**	**2.9**	**2.5**	**5.8**

Table 3.6: Relative fold changes for Cluster-IV. The genes that encode for the allophycocyananin and phycocyanain -the core light harvesting antennae belong to this cluster. All the *cpc* and *apc* transcripts are down-regulated and except for the genes encoding for phycobilisome degradation proteins *nb1A1* and *nblA2* that are up-regulated. The down-regulation of phycobilisomes is an important feature in photoprotection.

The figure 3.4 shows change in the expression ratios during various sampling points after exposure to light. All the genes show a down-regulation during the transition from low light to high light except for the gene encoding phycosobilisome degradation proteins (*nb1A)* that shows a steep up-regulation in the high light treatment (data not represented in the figure 3.4).

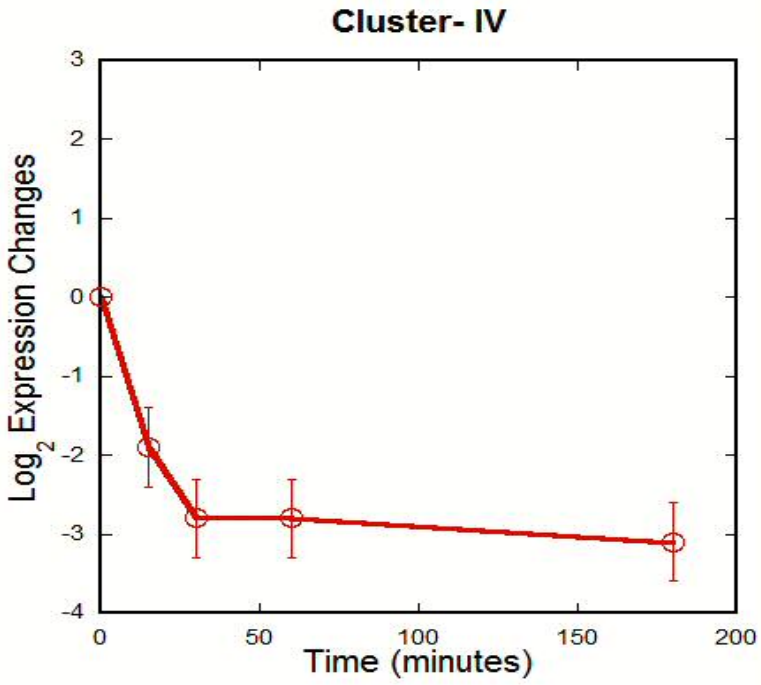

Figure 3.4: Overview for Cluster- IV. The *cpc* and *apc* transcripts the belongings to Cluster-IV are down regulated in the HL treatment. The X axis portrays the different sampling points after exposure to high light and the Y axis portrays the average log_2 expression ratios for the genes in this cluster.

Functional Cluster –V (Chlorophyll and Heme biosynthesis genes)

This cluster includes genes that transcribe enzymes required for the biosynthesis of photosynthetic pigments such as chlorophyll and heme. There is a net decrease in the chlorophyll content within the cell over time when exposed to high light. The transcripts of *chlB, chlL, chlN*, *por* encode for light-independent protochlorophyllide reductase subunits were down regulated during the shift from low light to high light. The transcripts of *ho1* were also down regulated, *ho1* encodes for heme oxygenase. Chlorophyll proteins are the major components of light harvesting antenna. Heme oxygenase acts as co-factor for the photosynthetic apparatus, it is also involved in regulating iron homeostatis. Heme oxygenase synthesizes biliverdin, precursor for phycobilins. Phycobilins are precursors for the chromophores found in phycobilisomes that act as light harvesting molecules.

Heme forms the prosthetic groups of cytochromes, these are engaged in the photosynthetic phosphorylation that generates ATP and NADPH. Since there was down-regulation of the phycobilisomes related genes, a similar trend was observed with the transcripts encoding for chlorophyll and heme biosynthesis subunits. The transcripts of *hem* genes were also down-regulated, *hem* genes are involved in the biosynthesis of tetrapyrrole. The transcripts of *isiA* that encodes for iron stress chlorophyll binding protein exhibited down-regulation after 60 minutes of exposure to high light. The function of *isiA* is not completely understood. But it seems to be up-regulated in oxidative stress and iron limiting conditions (Singh et al., 2003). The relative fold changes in this cluster are exhibited in table 3.7 and the over view of the cluster is displayed in figure 3.5.

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
slr0772	***chlB***	**-8.2**	**-5.9**	**-13.3**	**-8.9**
slr0749	***chlL***	**-2.7**	**-4.4**	**-9.7**	**-6.4**
slr0750	***chlN***	**-2.0**	**-2.3**	**-5.6**	**-4.2**
sll1091	***chlP***	**-8.6**	**-7.5**	**-7.0**	**-7.1**
slr1808	***hemA***	**-4.0**	**-4.3**	**-4.8**	**-5.0**
sll1994	***hemB***	**-1.4**	**-2.1**	**-1.5**	**-1.7**
sll1185	***hemF***	**-5.0**	**-4.1**	**-4.0**	**-4.2**
sll1184	***ho1***	**-28.1**	**-9.7**	**-18.9**	**-19.0**
slr0506	***por***	**-2.3**	**-2.4**	**-3.3**	**-3.4**
sll0247	***isiA***	**-1.2**	**0.8**	**-11.3**	**-4.6**

Table 3.7: Relative fold change for Cluster-V. The genes that encode for enzymes involved in chlorophyll biosynthesis and heme oxygenase were down-regulated on exposure to high light. The down-regulation was observed with in the initial 15 minutes of exposure to HL and remains the same through out the entire HL treatment. The negative sign for fold change indicates down-regulation.

The same observation as shown in the table 3.7 is depicted in the figure 3.5. All the genes that encode for chlorophyll biosynthesis and heme biosynthesis were down-regulated over time during the transition from low light to high light.

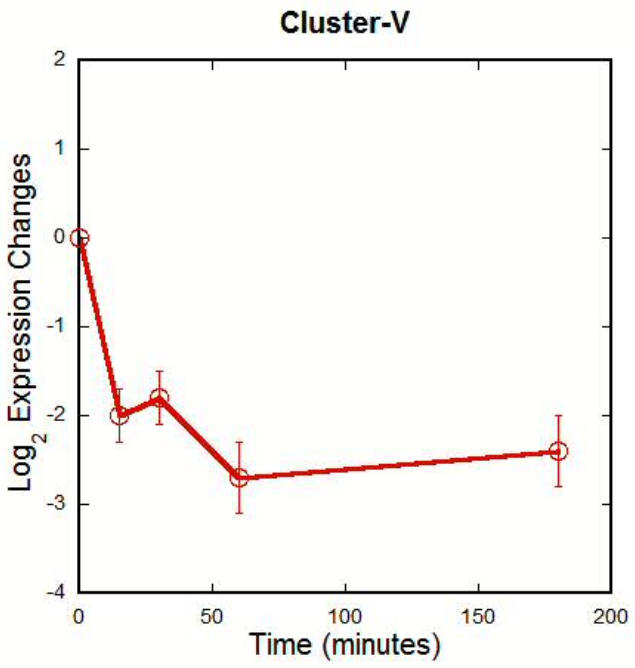

Figure 3.5: Over view of Cluster V. The chlorophyll related genes and the heme related genes were down-regulated. The X axis portrays the various time points after exposure to HL and the Y axis portrays the average log_2 values.

Functional Cluster –VI (Ribosomal related genes)

The genes belonging to this cluster encode for ribosomes (50S and 30S subunits) that are involved in translation of proteins. The transcripts of these genes were not induced at 15 minutes but were induced after 30 minutes. Some of the ribosomal genes were induced at 30 minutes and then decreased over time. This is not surprising that ribosomal genes are induced because the cells tend to divide faster when exposed to high light over time. The ribosomal operon *sll1799-sll1813* is actively induced at the same time point. The fold changes of this cluster are provided in table 3.8 and the overview of the cluster is depicted in the figure 3.6. Up-regulation of ribosomal genes is consistent with the increased growth rate observed under high light. Increase protein biosynthesis is dependent on ATP and NADPH and thus could be important for diminishing the negative effects of high light because the biosynthesis will absorb the products of the light

reactions. Consumption of ATP and particularly NADPH is important because the build-up of the end-product of light reaction can result in photoinhibition and oxidative stress.

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
sll1745	***rpl10***	**0.6**	**2.1**	**3.1**	**2.4**
sll1743	***rpl11***	**0.7**	**2.9**	**2.5**	**2**
sll1746	***rpl12***	**0.7**	**2.1**	**3**	**2.3**
sll1821	***rpl13***	**1.1**	**2.1**	**2**	**1.9**
sll1806	***rpl14***	**1.2**	**3**	**2.1**	**2.0**
sll1813	***rpl15***	**1.1**	**3**	**1.6**	**1.4**
sll1805	***rpl16***	**1.1**	**4.2**	**2.1**	**1.9**
sll1819	***rpl17***	**0.9**	**2.1**	**2.1**	**1.8**
sll1811	***rpl18***	**1.1**	**2.7**	**1.6**	**1.5**
sll1802	***rpl2***	**0.9**	**3.6**	**2.1**	**1.7**
sll1803	***rpl22***	**1.1**	**5.3**	**2.7**	**2.3**
sll1801	***rpl23***	**1**	**4.6**	**2.4**	**2**
sll1807	***rpl24***	**1.4**	**4.6**	**2.7**	**2.3**
sll1824	***rpl25***	**1**	**1.7**	**2.3**	**1.6**
ssl3436	***rpl29***	**1.3**	**6.7**	**2.5**	**2.2**
sll1799	***rpl3***	**1.0**	**4.1**	**2.2**	**2**
ssr1398	***rpl33***	**0.7**	**1.7**	**2.2**	**1.9**
smr0011	***rpl34***	**0.8**	**2.0**	**2.1**	**1.8**
sml0006	***rpl36***	**0.7**	**3.9**	**2.3**	**1.9**
sll1800	***rpl4***	**0.9**	**5.8**	**2.5**	**1.9**
sll1808	***rpl5***	**1.2**	**4.2**	**2.4**	**2.1**
sll1810	***rpl6***	**1.2**	**3**	**2.1**	**1.8**
sll1101	***rps10***	**1.0**	**1.9**	**2.4**	**1.9**
sll1817	***rps11***	**1.0**	**2.0**	**2.3**	**1.9**
ssl3437	***rps17***	**1.2**	**3.9**	**2.2**	**2.1**
ssl3432	***rps19***	**1.0**	**4.5**	**2.1**	**1.9**
ssl0601	***rps21***	**0.9**	**1.7**	**1.9**	**2.1**
sll1804	***rps3***	**1.1**	**3.9**	**2.3**	**1.9**
sll1812	***rps5***	**1.2**	**2.4**	**1.6**	**1.5**

Table 3.8: Relative fold change of Cluster VI. The genes encoding the 50S ribosomal subunits and 30S ribosomal subunits were actively up-regulated at 30 minutes and some of them continue to remain induced up to three hours.

The same tread can be observed in the figure 3.6 depicting change in the expression ratios over time. Most of the ribosomal genes indicate a two to six-fold up-regulation at 30 minutes and remain induced to two-fold for three hours during the transition from low light to high light. It is tempting to speculate that this up-regulation corresponds to increased ribosome biogenesis, but such a conclusion is premature especially in view of the absence of the information on the expression of the rRNA genes.

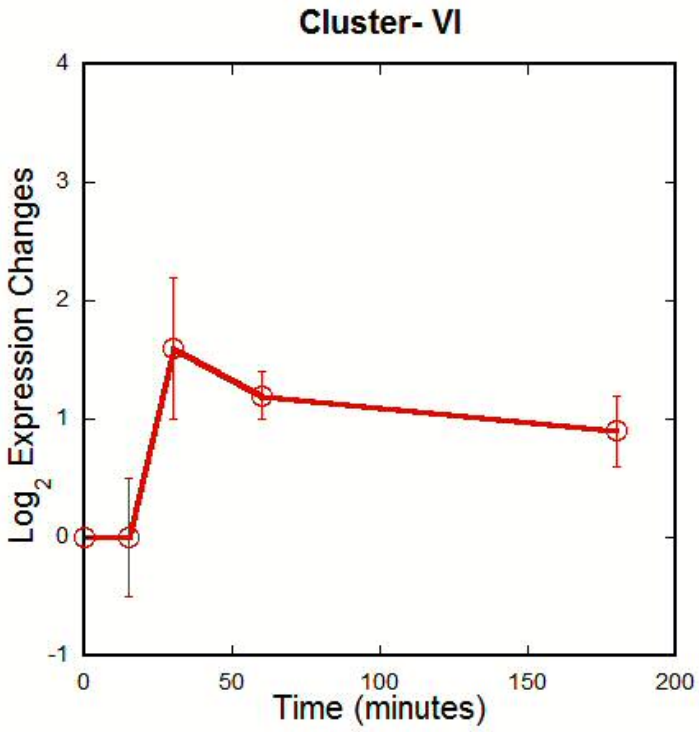

Figure 3.6: Overview of Cluster-VI. The transcripts for the ribosomal genes were accumulated after 30 minutes of exposure to high light. The X axis depicts the average log_2 expression ratios. The Y axis depicts the various time points after exposure to HL. There is no significant change at 15 minutes but there is almost a six-fold induction at 30 minutes of exposure to HL.

Functional Cluster-VII (High light inducible polypeptides)

This class of genes is highly expressed when cells are exposed to high light hence they are termed as *hli* genes (high light inducible polypeptides) or *scp* (small chlorophyll binding proteins). There are four genes that fall into this category of high light inducible polypeptides (*hliA, hliB, hliC, hliD*). Three of these genes are induced to high levels in our condition. The function of these genes is not completely understood, though they are proposed to play a major role in protecting the cells from damage caused by high light (Funk and Vermaas, 1999; He et al., 2001). They may be involved in facilitating the dissipation of excess absorbed energy and binding to the triplet chlorophyll molecules that are toxic to the cells. These charged chlorophyll molecules are released as a result of oxidative stress induced by high light. There were three ORFs that clustered with the high light transcripts after the hierarchical clustering. These include *slr1544*, *sll1483* and *sll1911*, the gene products of all theses ORFs are unknown or hypothetical. But it is known that *hliB* is physically clustered with *slr1544* and is predicted to encode for a membrane protein. These *hli* genes are also induced during nutrient stress and low carbon condition (Wang et al., 2004). The table 3.9 indicates the relative fold change in this cluster and the figure 3.7 depicts the overview of this cluster. The hypothetical proteins *sll1483* and *slr1544* was found to be co-purified with the Hli polypeptides in detergent extracts obtained from membranes of HL treated cells (Hsiao, 2005; personal communication).

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
ssl2542	***hliA***	**55.8**	**35.3**	**64.1**	**44.8**
ssr2595	***hliB***	**28.7**	**8.9**	**20**	**15.8**
ssl1633	***hliC***	**26.6**	**37.0**	**42.2**	**33.1**
sll1483	***hypothetic***	**38.6**	**41.5**	**102.3**	**65.6**
slr1544	***unknown***	**17.3**	**12.9**	**9.1**	**7.9**
sll1911	***hypothetic***	**14.9**	**9.4**	**21.3**	**14.1**

Table 3.9: Relative fold changes for Cluster-VII. The *hli* genes respond early when exposed the HL. The hypothetical ORFs that cluster along with *hli* is also represented here.

The figure 3.7 indicates the overview of the genes belonging to Cluster VII and the transcripts of all the genes in this cluster are induced at high levels during the exposure to HL.

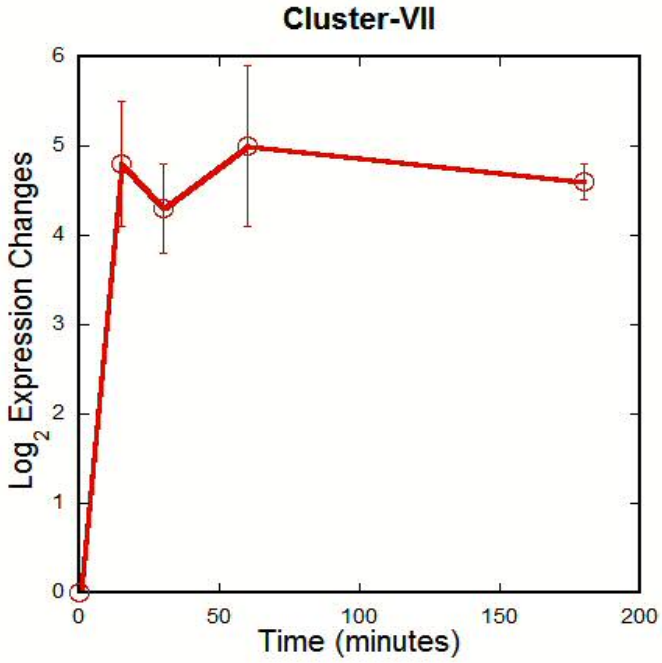

Figure 3.7: Overview for Cluster-VII. The X axis indicates is the average $\log_2$ expression ratios. The Y axis indicates the sampling time points after exposure to HL. All the genes in this cluster are induced to high levels. This cluster includes the *hli* genes and the unknown ORFs such as *sll1911*, *sll1483*, *slr1544*.

Functional Cluster –VIII (PSII repair genes)

This is the cluster of genes that are hypothesized to play a role in PSII repair. The role of FtsH proteases in D1 repair has been investigated. The FtsH protease involved in the repair mechanism in *Synechocystis* sp. PCC6903 is *ftsH (slr0228)* (Imamura et al., 2004; Nixon et al., 2005). While *ftsH* (*slr1604*) is important for cell viability but the exact function in *Synchocystis* sp. PCC6903 is not completely understood. These *ftsh* homolgs in plants is known to be involved in D1 turnover (Nixon et al., 2005). These proteases are transmembrane proteins, involved in the removal of damaged D1 subunits. In high light the D1 turnover rate is also increased hence these genes are induced to high levels. The *psbA2* and *psbA3* encode for D1 protein and as mentioned earlier these genes are induced in high light to facilitate D1 repair. The sigma factor D is said to be involved in the promoter recognition of *psbA2* (Imamura et al., 2004). Hence it is also induced so as to assist in D1 repair process. The heat shock genes and the molecular chaperonins are predicted to play a major role in D1 repair because they are responsible for folding new proteins and refolding old proteins. The other genes include peroxidases that protect the cells from oxidative stress such glutathione peroxidase were also seen to be induced in the high light treatment. The *gro-EL* is a 60kDa molecular chaperone was induced through out the high light treatment and evidently it is shown to be induced during general stress response. The fold changes for the genes belonging to this cluster are provided in table 3.10 and the cluster view are also displayed in figure 3.8.

ORF	Product	Fold change 15min	Fold change 30min	Fold change 60min	Fold change 180min
slr1604	***ftsH***	**5.9**	**3.5**	**2.8**	**2.1**
slr0228	***ftsH***	**6.9**	**4.3**	**3.3**	**2.7**
sll2012	***sigD***	**8.9**	**6.4**	**5.8**	**5.6**
sll0416	***groEL-2***	**12.6**	**4.4**	**8.7**	**6.6**
slr1311	***psbA2***	**1.7**	**1.8**	**3.7**	**3.8**
sll1867	***psbA3***	**2.5**	**3.9**	**6.8**	**6.4**

Table 3.10: Relative fold changes in Cluster-VIII. The Cluster-VIII includes the PSII repair genes that include the two *ftsH* proteases *slr1604* and *slr0228*, *sigD*, *psbA2*, *psbA3*, and *groEL* a 60kDa molecular chaperonin. The *ftsH* (*slr1604*) may also play a role in the repair process because it is induced during HL stress (Hihara et al., 2001; Tu et al., 2004), redox stress (Hihara et al., 2003) and low carbon stress (Wang et al., 2004)

The Figure 3.8 also illustrates the same trend as observed in the Table 3.11, all the PSII repair genes are induced to high levels.

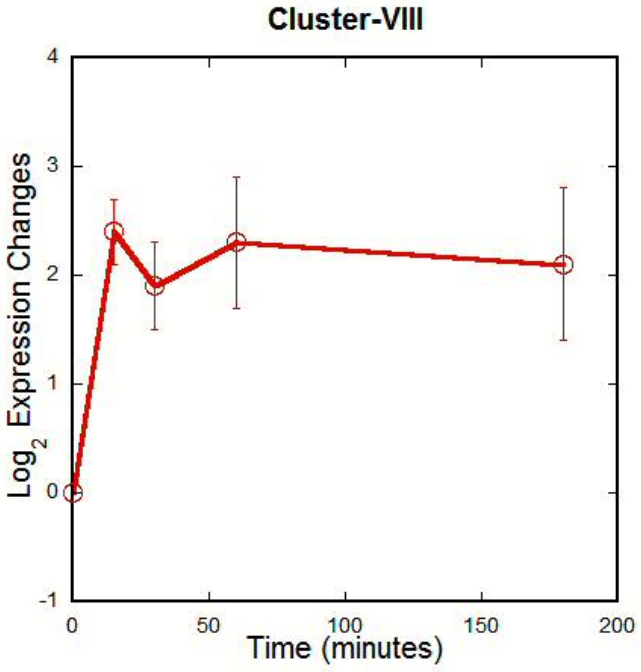

Figure 3.8: Overview of Cluster-VIII. Cluster-VIII includes the transcripts that belong to the PSII repair regulon. The transcripts were up-regulated to four-fold within 15 minutes after exposure to HL. The transcripts remain up-regulated during the entire course of high light treatment.

Changes in transcript abundance of other genes during the transition from low light to high light

There were genes that not classified into the clusters defined earlier, but these genes were induced or repressed as a result of high light treatment. Among the genes that were induced within 15 minutes of exposure to HL was the *ccmK-N* operon. These genes encode for carbon concentrating mechanism (carboxysomes). The transcript level of *ccmK* (*slr1839*) and its homolog another *ccmK* (*slr1838*) were both induced to 4.8-fold and 4.4-fold respectively, *ccmL*, *ccmM* and *ccmN* were also induced to 3.9-fold, 2.6-fold and 3-fold respectively after 15 minutes of exposure to HL, but the transcripts abundance of these genes were subsequently reduced after 60 minutes of exposure to HL. The transcript for the *ndhD2* that encodes for NDH dehydrogenase subunit 4 was up-regulated to high levels during the high light stress. The *ndhD2* is essentially involved in the synthesis of the respiratory NDH-1 complex and this complex is involved in the electron transport. The transcripts of *rbcx* responsible for synthesis of RbcX chaperone for rubisco were also seen to be transiently accumulated at 15 minutes of high light exposure.

Another cluster of genes that were transiently up-regulated were the nitrate transporters, *nrtA*, *nrtB*, *nrtC*, *nrtD* and *urtD* these form a transcriptional unit. The transcripts of *urtD* did not show any distinct change in expression but the transcripts of the genes *nrtA*, *nrtB*, *nrtC*, *nrtD* were up-regulated at 15 minutes but exhibited relatively lower abundance at 60 minutes and 180 minutes of exposure to HL. The sigma factors that displayed significant changes in their expression patterns were *sigA*, *sigB*, *sigD*, *sigG*, of these *sigD* was grouped in the cluster that represents the hypothetical PSII repair regulon. The *sigG* exhibited lower transcript abundance through out the high light

treatment, where as *sigB* was shown to be induced to 6.9-fold at 15 minutes of exposure to HL but fail to remain up-regulated at later time points, the transcripts of *sigA* was also moderately down-regulated. There was an initial induction of *sobB* (*slr1516)* which encodes for the ROS scavenging enzyme superoxide dismutase, at 15 minutes of exposure to HL but ceased to be induced at high levels at later sampling points. The transcripts of *gpx1* were also transiently induced and transcripts responsible for caroteniods were seen to be up-regulated these include *slr1254, slr1963*. Carotenoids are involved in photoprotection by quenching singlet oxygen species generated due to oxidative stress (Rakhimberdieva et al., 2004). The transcripts of *ctpA* that are involved in the synthesis of carboxyl terminal processing protease, vital for the processing D1 precursor was also shown to accumulate in HL. The two component response regulators that include *slr0312*, *sll0797*, *sll1330, sll0798 (hik30), slr0533 (hik10), slr1324 (hik23*) exhibited down-regulation during the transition from LL to HL.

Conclusion

The gene expression patterns using microarrays demonstrate the responses of the photosynthetic organisms during the shift from low light to high light. The changes in transcript levels do not reflect the changes in the amount of corresponding proteins nor does it indicate whether the transcripts are degraded over time. There were 3168 ORF's of *Synechocystis* sp. PCC6803 included in the microarray analysis. Interestingly, when expression changes are mapped out according to their physical location on the *Synechocystis* sp. PCC6803 chromosome, adjacent genes were coordinately up-regulated or coordinately down-regulated. In some cases, this corresponds to experimentally defined operons, whereas in many cases the possibility of operonic structure can only be hypothesized at this point. The genes were classified as functional clusters based on supervised clustering and for this purpose, the genes that exhibited two-fold change for at least one time point were considered. The ORFs were grouped into eight different functional categories. The functional clusters are described in detail in the earlier sections. The PSII repair regulon defined as the hypothetical cluster consists of the following genes *psbA2 (slr1311), psbA3 (sll1867), sigD (sll2012), ftsH (slr1604), ftsH (slr0228), groEL-2.* The *groEL-2* is member of the cluster –I that includes heat shock proteins and these proteins are induced as a result of a general stress response. The g*roEL-2* was included in the PSII repair regulon based on its pattern of induction, it seems to be induced to same extent throughout the treatment and levels of induction are similar to other members of the PSII repair regulon (table 3.10). High light is a typical stress that could activate the chaperones for folding and unfolding proteins, as protein folding is commonly affected during heat and light stress. The other members of the

repair regulon include *sigD*, as described earlier this particular sigma factor is known to regulate the transcription of *psbA2,* but microarray analysis of redox, oxidative and nutrient stress and the data shown in this chapter (HL stress) indicates that this sigma factor plays a definite role in PSII repair. The *psbA2*, *psbA3* are regulated by change in the redox state and this have been investigated by various researchers (El Bissati and Kirilovsky, 2001; Imamura et al., 2003a). It is not surprising though that these genes are also affected in this HL experiment because HL induces a redox stress caused by increased flux of photons. The photoinhibition is commonly observed phenomenon in HL, there are various levels of photoprotection. One of them as described earlier is down-regulation of the light harvesting antenna (phycobilisomes), down-regulation of chlorophyll related genes such as genes regulating chlorophyll biosynthesis and heme synthesis. Down-regulation of PSII and PSI subunits were observed except for the core PSII proteins such as D1and D2 that were up-regulated. There is an increase in PSII/PSI ratio, the PSI subunits are more severely down-regulated than PSII. The up-regulation of high light inducible polypeptides indicates that they play an important role in photoprotection and may be involved in binding to the free chlorophyll molecules. Both *ftsHs* were induced suggesting that they play a role in photoprotection by facilitating the removal of damaged D1. The genes encoding for caroteniods are also induced to protect against oxidative damage by quenching reactive oxygen species generated by HL (Rakhimberdieva et al., 2004). SigD being a part of the repair regulon and since it regulates *psbA2* in HL, it was interesting to understand its regulatory mechanism in HL. "Does SigD regulate other members in the PSII repair regulon" was the objective of my thesis discussed in later sections.

B. Comparison of microarray results between WT with *Synechocystis* SigD and *Synechocystis* SigD^c

In *Synechocystis* SigD mutant stain, the *sigD* gene was rendered in active by insertion of kanamycin cassette. This mutant strain was contributed by Dr. Louis Sherman (Purdue University). The *Synechocystis* SigD^c mutant strain was constructed as described in materials and methods section. This mutant was capable of constitutively expressing SigD and this gene was under the control of a petE promoter. The *sigD* is a key component of PSII repair regulon and this experiment was done to test the importance of *sigD* in light regulation of PSII repair genes. The hybridizations were set up as done earlier for WT explained in the earlier sections. The hybridizations that were set up for the SigD and SigD^c are described in table 3.11

Strains	Hybridizations	Dyes used
Synechocystis SigD	SigDLL- SigDHL15min	Alexa555 vs. Alexa647
	SigDLL- SigDHL15min	Alexa647 vs. Alexa555
	SigDLL- SigDHL30min	Alexa555 vs. Alexa647
	SigDLL- SigDHL30min	Alexa647 vs. Alexa555
	SigDLL- SigDHL60min	Alexa555 vs. Alexa647
	SigDLL- SigDHL60min	Alexa647 vs. Alexa555
	SigDLL- SigDHL180min	Alexa555 vs. Alexa647
	SigDLL- SigDHL180min	Alexa647 vs. Alexa555
Synechocystis Wild Type and *Synechocystis* SigD	WTLL- SigDLL	Alexa555 vs. Alexa647
	WTLL- SigDLL	Alexa647 vs. Alexa555
Synechocystis Wild Type and *Synechocystis* SigD^c	WTLL- SigD^{c}LL	Alexa555 vs. Alexa647
	WTLL- SigD^{c}LL	Alexa647 vs. Alexa555

Table 3.11: The list of microarray hybridizations for SigD and SigD^c. The hybridizations were performed twice with a biological replicates.

SigD is known as the high-light-response sigma factor and one of the major goals of this work was to understand the possible promoters recognized by SigD. The *sigD* plays a role of the PSII repair regulon. This has been established from the microarray data

with WT in HL as shown in section A of this chapter. But it is also evident from the various studies done in the presence of redox agents, hydrogen peroxide, iron limiting and carbon limiting conditions and also HL stress (Imamura et al., 2003a; Singh et al., 2004). To identify other genes that would play a prominent role in PSII repair DNA microarrays was used as a tool to test this hypothesis. The strains that were used for this study were the *Synechocystis* Wild Type, *Synechocystis* SigD mutant and *Synechocystis* SigD^c mutant. In order to compare SigD mutant with the WT, both the strains were grown identically and induced with HL stress in the same manner. Table 3.12 illustrates the correlation between the different biological replicates in SigD mutant strains.

Hybridization	Correlation between the biological replicates
SigDLL- SigDHL15min	0.7318
SigDLL- SigDHL30min	0.8916
SigDLL- SigDHL60min	0.9172
SigDLL- SigDHL180min	0.9269

Table 3.12: Correlation coefficient between the biological replicates for SigD mutant. The higher the correlation coefficient the greater is the similarity between the biological replicates. The table shows the correlation coefficient for the different sampling time points in the SigD mutant strain during the transition from LL to HL.

To recognize any variation in gene expression in LL between the WT and SigD mutant, the hybridization between WTLL and SigDLL was also performed. This would help to compare the difference in gene expression between the WT and SigD mutant. The constitutively expression of SigD mutation that was constructed as described in materials and methods section was also analyzed using microarrays. The hybridization between WTLL and SigD^cLL was also performed.

The threshold hold for significance was based on p-value < 0.05 (false discovery rate) for at least one time point. There were 3168 genes or ORFs of *Synechocystis* sp. PCC6803 spotted on the arrays including three replicate spots for each gene and two biological replicates for each time point. There were 393 ORFs that exhibited at least two-fold change or greater in at least one of the four sampling time points. The hierarchical cluster analysis was conducted to compare the WTLL-WTHL versus SigDLL-SigDHL. Surprising the genes in the WT and the SigD mutant exhibited almost similar expression pattern. There were no striking differences observed between the SigD mutant and the WT. There were only a handful of genes in SigD mutant that exhibited different expression pattern compared to the WT. The genes that were included in the analysis were *psbA2*, *psbA3, ftsH* (*slr1604*), *ftsH* (*slr0228*), *slr1963*, *groEL-2*, *psbD*, and *psbD2*. The expression patterns of these genes are indicated in figure 3.9. The $\log_2$ differential expression ratio for this cluster of genes is illustrated in table 3.13. The over view of this cluster is represented in figure 3.10. Comparing the trend in gene expression the between the SigD mutant and the WT for this cluster (figure 3.9), it can be seen that these genes were not induced to high levels in the SigD mutant as compared to the WT. The *sigD* gene is induced to high levels in the WT within 15 minutes of exposure to HL but as *sigD* was inactivated in the mutant, *sigD* was not expressed in the mutant (figure 3.9).

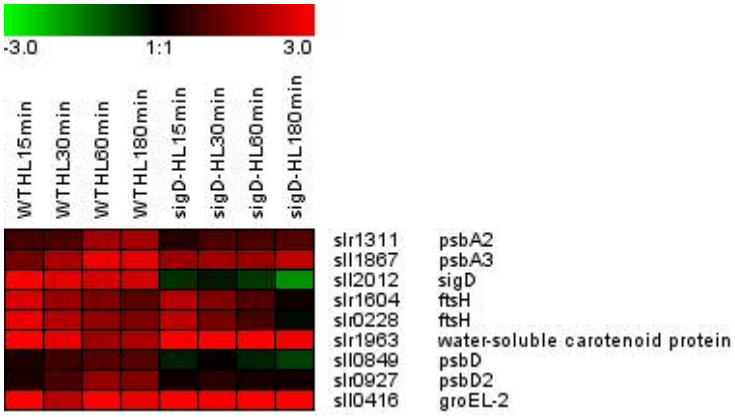

Figure 3.9: The cluster of genes that differ between the SigD mutant and the WT. The red color indicates up-regulation and green color depicts down-regulation. This figure represents the expression pattern of the genes in the cluster. The figure is represented in $\log_2$ based scale.

Table 3.13: $\log_2$ differential expression ratios for the cluster of genes in the SigD mutants and WT.

		$\log_2$ Differential Expression ratios							
ORF	**Product**	**WTHL 15min**	**WTHL 30min**	**WTHL 60min**	**WTHL 180min**	**SigDHL 15min**	**SigDHL 30min**	**SigDHL 60min**	**SigDHL 180min**
slr1311	***psbA2***	**0.80**	**0.88**	**1.87**	**1.94**	**0.41**	**0.85**	**0.87**	**0.95**
sll1867	***psbA3***	**1.34**	**1.95**	**2.77**	**2.69**	**1.86**	**1.90**	**1.80**	**2.27**
sll2012	***sigD***	**3.15**	**2.67**	**2.53**	**2.48**	**-0.60**	**-0.31**	**-0.69**	**-1.74**
slr1604	***ftsH***	**2.57**	**1.83**	**1.51**	**1.04**	**2.14**	**1.53**	**0.96**	**0.17**
slr0228	***ftsH***	**2.79**	**2.09**	**1.70**	**1.45**	**2.21**	**1.38**	**0.85**	**-0.15**
slr1963	***water-soluble carotenoid protein***	**3.50**	**2.75**	**1.87**	**2.01**	**3.67**	**3.70**	**3.24**	**2.85**
sll0849	***psbD***	**0.32**	**0.79**	**1.00**	**0.96**	**-0.37**	**0.06**	**-0.41**	**-0.76**
slr0927	***psbD2***	**0.33**	**0.85**	**1.75**	**1.53**	**0.16**	**0.46**	**0.22**	**0.18**
sll0416	***groEL-2***	**3.66**	**2.14**	**3.12**	**2.72**	**4.23**	**2.92**	**3.37**	**3.34**

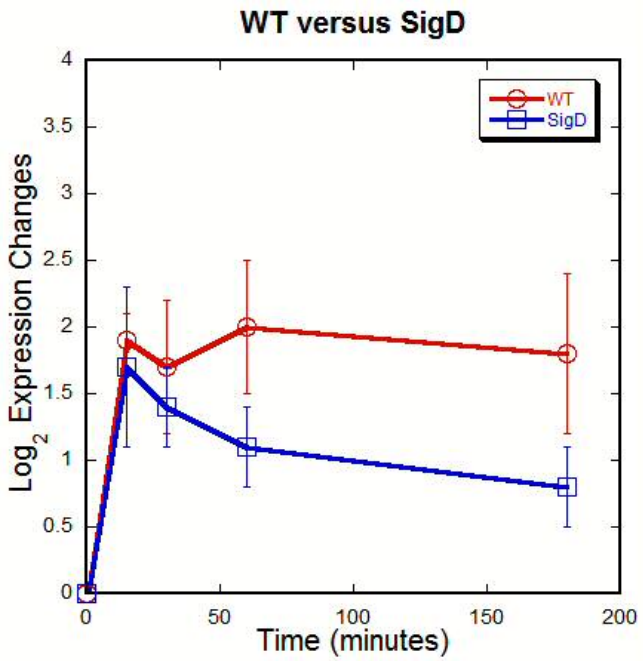

Figure 3.10: Over view of the genes that belong to the cluster in WT and the mutant**.** The genes in this cluster are induced in the WT but in the mutant it is not induced to high levels as the WT. The X axis depicts the $\log_2$ expression ratios ad the Y axis s the various sampling points under HL. The genes included in this graph are *psbA2*, *psbA3*, *ftsH* (*slr1604*), *ftsH* (*slr0228*).

The *psbA2* transcripts exhibit 3.7-fold up-regulation after 60 minutes of exposure to HL and remains the same after 180 minutes of exposure to HL, on the other hand in the SigD mutant there isn't any distinct change in *psbA2* gene expression as observed from figure 3.9 The *psbA3* transcripts are up-regulated to 2.5-fold at 15 minutes to 6.4-fold after 180 minutes of exposure to HL in the WT but in the mutant the transcripts are up-regulated to 3.6-fold and increases to 4.8-fold after 180 minutes of HL treatment which is at least two-fold less than the WT. The *ftsH* (*slr1604*) and *ftsH* (*slr0228*) were induced to almost six to seven-fold within 15 minutes of exposure to HL but decreased to two-fold after 180 minutes in the WT but in the SigD mutant these genes were initially induced to two-fold but fail to remain up-regulated through out the HL treatment (table

3.13, figure 3.9). The genes encoding the PSII core proteins D2 also show the similar trend. These genes were not induced in the SigD mutant as much as they were induced in the WT. The interesting fact is though that the genes encoding water soluble carotenoid proteins and the 60kDa molecular chaperonone (*groEL-2*) are induced at much higher levels in the SigD mutant than in the WT. The gene encoding the molecular chaperonone *groEL*-2 was induced to 18-fold within 15 minutes of exposure to HL in SigD mutant and remains induced at much higher levels than the WT. The ORF *slr1963* is also induced at much higher levels in the SigD mutant than the WT.

The carotenoids are required to quench reactive oxygen species, since it is induced at higher levels in the mutant, it could be suggested that the SigD mutant was experiencing a more pronounced oxidative stress compared to the WT. Since the mutant was exhibiting relatively lower expression of *psbA2*, it could be proposed that SigD mutant was succumbed to photoinhibition after long term exposure to HL and may have an impaired repair mechanism after prolonged exposure to HL as the *ftsH* genes also fail to remain up-regulated in the SigD mutant. This may suggest that the disruption of *sigD* gene in the mutant hampers the expression of *psbA2,* and *ftsH (slr0228)* and *ftsH (slr1604).*

The hybridization WT versus SigD^{c} was done only using LL condition because SigD was high light induced sigma factor and since its being constitutively expressed in SigD^{c} mutant. This constitutive expression of *sigD* could induce the genes that are regulated by SigD. But interestingly results demonstrated only *sigD* transcripts were up-regulated to eight-fold and none of the other genes that were assumed to be a part of a repair PSII repair regulon including *psbA2, psbA3*, *ftsH* (*slr1604*) or *ftsH* (*slr0228*)

displayed any distinct changes in gene expression. Though *sigD* is induced constitutively in the SigD^{c} mutant but abundance of protein was not tested. There may other transcriptional factors could limit the activity of SigD under low light conditions.

Earlier studies also suggest that the structural changes to SigD may be caused by redox signal along with other *trans* –acting factors enhances the sigma binding affinity to the promoter or the core enzyme (Imamura et al., 2003a). Studies have also indicated that *sigD* transcripts are also reduced in ΔHik33 (a sensor histidine) (Imamura et al., 2003a) . This indicates that sensor protein may be involved in the expression of *sigD* (Imamura et al., 2003a). This could be true because in this work with SigD^{c} mutants the genes that SigD regulates are not turned on in absence of light or redox stress.

Does SigD regulate the genes in the repair regulon?

Changes in the transcriptional abundances of the PSII repair genes in the SigD mutant when compared to WT appears to be rather small and become more pronounced at later time points. In stress conditions the sigma factors are recruited for the transcription of different genes, SigD seems to be solely recruited for the transcription of the *psbA* genes. But in the SigD mutant, due to the absence of this sigma factor, the up-regulation in the *psbA* transcripts is not high as seen in the WT and it appears that other sigma factors are not able to substitute for SigD. Given the redundancy with primary sigma factor, SigA, I propose that SigD supplements SigA under conditions of high transcriptional activity.

Studies done by other groups in our lab also suggests that *psbA* transcript tends to be in excess in cyanobacterial cells hence physiological phenotype can be observed only under extreme conditions and this has not been exploited. Transcription of *psbA2* is also

regulated by enhancers (Li and Golden, 1993; Takahashi et al., 2004). The *psbA* genes are regulated by enhancers as well the sigma factor. Hence during stress conditions, sigma factors become limiting and in the WT under HL, SigD along with enhancers up-regulate the transcription of the *psbA* genes and hence these genes are dramatically up-regulated in the WT when compared to the SigD mutant. Hence despite the presence of enhancers in the SigD mutant, *psbA* genes fail to remain up-regulated.

C. Comparison of the high light response between WT grown in air versus WT grown in air supplemented with 3%CO2

The goal of this experiment was to understand the function of PSII repair regulon in HL stress when cells were grown under two different growth conditions. Another important objective was to emphasize the discrepancies observed in earlier papers describing "DNA microarrays analysis to understand the cyanobacterial gene expression during acclimation to HL" (Hihara et al., 2001). The authors of this paper had detected a particular subset of genes that were induced in their HL treatment. The genes that seem to be induced in their experiment were *ndhF3*, *ndhD3*, *cupA* and *sll1735* (hypothetical). Theses genes function as an multicistronic operon and are induced during carbon limiting conditions (Wang et al., 2004). In the HL experiment discussed earlier with WT grown in air supplemented inorganic carbon (bubbled with 3% CO_2) these high affinity inorganic carbon (Ci) uptake transporters were not induced. This was also noticed in experiments done using WT in HL (Tu et al., 2004). The authors of this paper compared the gene expression profiles of the WT and *dspA* deletion mutants in HL stress. The high affinity uptake system for inorganic carbon was not induced in their HL treatment as well. The objective was to compare and contrast the HL experiment discussed in earlier section with Hihara et al's 2001 HL experiment. Another objective was to verify whether the PSII repair regulon is up-regulated to the same extent as in the HL treatment or is it induced to higher levels when the cells are grown in moderate Ci and subjected to HL. The microarray hybridizations were set up as follows with WTLL air grown as the reference and WTHL air grown as the treatment (table 3.14).

Strains	Hybridizations	Dyes used
Synechocystis Wild Type	WTLL-WTHL15min	Alexa555 vs. Alexa647
	WTLL-WTHL15min	Alexa647 vs. Alexa555
	WTLL-WTHL30min	Alexa555 vs. Alexa647
	WTLL-WTHL30min	Alexa647 vs. Alexa555
	WTLL-WTHL60min	Alexa555 vs. Alexa647
	WTLL-WTHL60min	Alexa647 vs. Alexa555
	WTLL-WTHL180min	Alexa555 vs. Alexa647
	WTLL-WTHL180min	Alexa647 vs. Alexa555
Synechocystis Wild Type	Air-WTLL-Air-WTHL15min	Alexa555 vs. Alexa647
	Air-WTLL-Air-WTHL15min	Alexa647 vs. Alexa555
	Air-WTLL-Air-WTHL30min	Alexa555 vs. Alexa647
	Air-WTLL-Air-WTHL30min	Alexa647 vs. Alexa555

Table 3.14: The hybridizations for HL response in WT grown in moderate Ci and supplemented Ci. The hybridizations were performed in replicates.

Microarray analysis of high light response in the WT cells grown in air

Hybridization	Correlation between the biological replicates
Air-WTLL-Air-WTHL15min	0.954
Air-WTLL-Air-WTHL30min	0.8216

Table 3.15: The correlation between biological replicates in the HL treatment in air grown WT. Higher the correlation the greater is the similarity between the replicates other

The cut-off for significance was based on p-value < 0.05 (false discovery rate) for at least one time point. There are 3168 genes or putative ORFs of *Synechocystis* sp. PCC6803 and 563 ORFs consistently displayed expression changes of at least two-fold or greater ($\log_2 < -1$ or $\log_2 > 1$) for at least one of the two sampling time points (15 minutes and 30 minutes) .The ORFs clustered as the WTLL –WTHL experiment that has been discussed in section A of this chapter. Based on their expression pattern and their function, the ORFs were classified into different functional groups that were termed as

functional clusters. The synchronized expression pattern (up-regulation or down-regulation) of genes could be associated with an operon structure, in other words these genes are physically clustered together on the chromosome and is shown to be regulated in the same way.

PSII repair regulon

The genes were divided into the same clusters as described earlier for the WTLL-WTHL experiment. While comparing the genes that belong to PSII repair regulon between the WT grown in air and CO_2 in HL response. The transcripts of *ftsH (slr1604)* were induced to very high levels within 15minutes of exposure to HL for the cultures grown in air. The transcripts for *ftsH* (*slr1604*) was induced to 5.9-fold within 15 minutes of exposure to HL for the cultures grown in air supplemented with 3% CO_2 whereas for cultures grown in air alone it was induced to 11-fold within 15 minutes of exposure to HL. Similar observation was noticed for transcripts of *sigD* and *gro-EL*, both were induced to 9-fold and 25-fold within 15 min of exposure to HL respectively in the cultures bubbled with air. The transcripts of *psbA2*, *psbA3*, *ftsH* (*slr0228*) and *groEL* seem to be induced to the same levels in both the conditions. The over view of the genes in this cluster is depicted in figure 3.11.

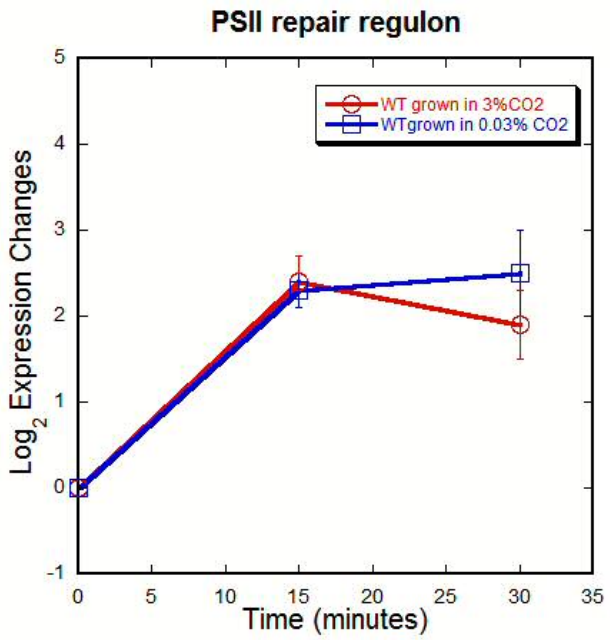

Figure 3.11: Comparison of the genes in PSII repair regulon in the high light response in WT cultures grown in the two conditions

The genes *psbA2, psbA3* encode for the D1 protein that forms the core of photosystem II and this protein is revealed to undergo rapid turnover. This turnover rate is much higher during photodamage. Earlier studies done under carbon limiting condition have also revealed that these genes are induced, because of increased photodamage, the reason for the photodamage being increased reduction of the electron transport chain, due to reduced availability of electron acceptor (inorganic carbon) for the removal of electrons generated by PSII (Wang et al., 2004). Surprisingly *ftsH* (*slr1604*) is induced much higher levels in cells grown in moderate inorganic carbon exposed to HL. This is because *ftsH* is known to be involved in the repair of D1 in higher plants. FtsH is a transmembrane protease that is involved in proteolytic removal of photodamaged D1 from PSII (Nixon et al., 2005). Both the *ftsHs* have shown to be low carbon as well redox responsive genes (Hihara et al., 2003; Wang et al., 2004). The *ftsH* (*slr0228*) is

involved in the removal of damaged D1 in *Synechocystis* sp. PCC6803 (Nixon et al., 2005).

The plausible explanation for the transcripts encoding heat shock proteins and chaperonins such as *hspA*, *htpG*, *groEL*, *groEL2*, *dnaK1* and *dnaK2* being up-regulated, because HL induces photodamage and this could damage not only the PSII core proteins D1 and D2 but also other proteins, hence these genes were induced to protect against photodamage, however these genes are not directly involved in the photosynthetic activity. These genes seem to be repressed during carbon limitation stress and in the presence of inhibitors such as DCMU (Hihara et al., 2001; Wang et al., 2004). The heat shock proteins are highly expressed in response to elevated temperatures. This is because protein folding is severely affected due to high temperature and these molecular chaperones are recruited to repair the damage caused by misfolding. The same would apply to HL damage, the proteins are damaged and these chaperonins are engaged to assist in repairing the damage caused by HL.

The only transcription factor that seems to play a major role in PSII repair sigma factor D. The transcript abundance of *sigD* is shown to be elevated in redox stress, carbon limitation condition and HL stress. SigD has been implicated in the regulation of *psbA2* during HL and redox stress (Imamura et al., 2003b).This is very clear with the microarray analysis of HL data that was described in detail in the earlier sections. Here the results confirm the same, *sigD* was induced to high levels within the initial 15 minutes of exposure to HL in WT cultures grown in air.

PSII repair process seems to be a well coordinated process with many genes playing an important role in photoprotection, it includes a subset genes that are not

directly involve with photosynthetic activity but do play a prominent role in protecting against photodamage. These include the *hli* genes, the function of these genes is not completely understood but has been demonstrated that the expression of these genes is crucial during nutrient stress, HL and redox stress.

The genes encoding the heat shock proteins and chaperonins are involved in folding of new proteins and refolding of old proteins. The genes such as *gpx2* and *gpx1* encoding for glutathione peroxidases, genes encoding for carotenoid proteins also seem to be up-regulated to protect against oxidative stress which is a common feature in HL.

Phycobilisomes related genes

The genes belonging to this cluster includes the *apc* and *cpc* and these genes are down-regulated in HL. The down-regulation is more pronounced in WT cells grown in air rather than the cells grown in 3% CO_2, this can be observed from the figure 3.12

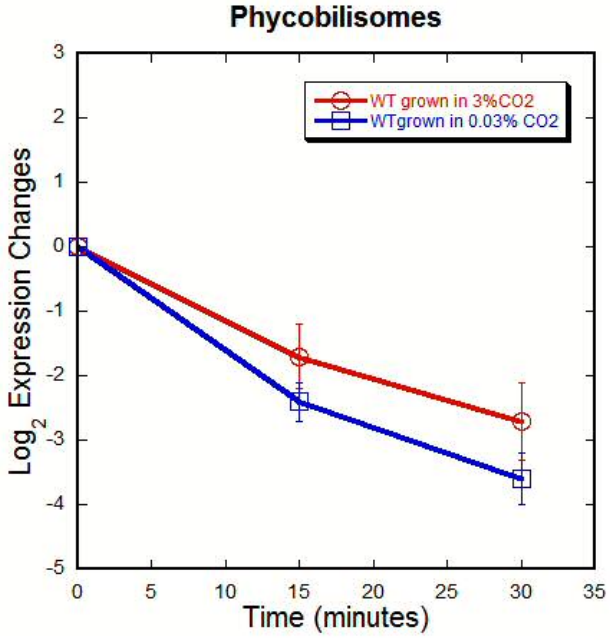

Figure 3.12: Over view of the Phycobilosomes related genes in both the growth conditions. The repression seems to be more pronounced in moderate inorganic carbon condition rather than the supplemented inorganic carbon condition after exposure to HL

Carbon Concentration Mechanism (CCM)

Inorganic carbon (Ci) is often a limiting substrate in oxygenic photosynthesis, especially under high light (HL) conditions. This limitation is due, in part to fact that the main Ci-fixing enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco) has a low affinity for CO_2 and thus requires high ambient levels of CO_2 to maintain adequate rates of Ci fixation into organic products used in metabolism. In order to increase intracellular concentration of CO_2, cyanobacteria have evolved complex inorganic carbon concentrating mechanism (CCM). There are two major major inorganic carbon transport systems found in *Synechocystis* sp. PCC6803*,* the high affinity inorganic carbon transporters and the low affinity inorganic transporters.

The high affinity inorganic carbon uptake transporters are induced during inorganic carbon limitation. The mechanism of induction of these genes remains to be fully characterized, however considerable information on the identity of the important genes has been made during the last decade. The high affinity transport system consists of three different protein complexes.

1. The *sbtA* operon includes *sbtA* (*slr1512*) and *sbtB* (*slr1513*), this is a sodium dependent bicarbonate transporter
2. The *cupA* operon includes *ndhF3* (*sll1732*), *ndhD3* (*sll1733*), *cupA* (*sll1734*) and *hypothetical* ORF (*sll1735*). This operon functions as a multicistronic operon and the gene product is a single transcriptionl unit. These genes encode a NADPH dependent CO_2 transporter.

3. The *cmpABCD* is also a multicistronic operon that includes *cmpA* (*slr0040*), *cmpB* (*slr0041*), *cmpC* (*slr0043*), *cmpD* (*slr0044*) and these genes encode an ATP dependent bicarbonate transporter.

The high affinity system supplements the activity of another group of transporters that are constitutively expressed and are collectively known as the low affinity inorganic carbon uptake transport system. As with the high affinity system is composed of more than one type of transport protein, this group includes *ndhF4* (*sll0026*), *ndhD4* (*sll0027*) and *cupB* (*slr1302*).

There is an interaction between the regulation of the genes encoding the proteins of the light reactions and the regulation of genes encoding proteins for Ci uptake and fixation (light-independent reactions) given the close physiological interplay between the reactions catalyzed by the light and light-independent processes of photosynthesis. However, a thorough analysis of the regulation of the high affinity CCM genes in relation to the prevailing light conditions remains to be performed. In this section I would like to note out the discrepancies between the Hihara et al 2001 microarray data and the microarray HL data in the two different growth conditions discussed earlier. Hihara et al 2001 examined the gene expression in *Synechocystis* sp. PCC6803 during the acclimation to high light, and have stated that in their HL treatment they observed the high affinity inorganic carbon uptake system being up-regulated. The ORFs that were induced included *sll1732*, *sll1733*, *sll1734* and a functional explanation for the induction of these high affinity CO_2 uptake transporters is that since CO_2 fixation is increased in HL due to the increased rate of ATP and NADPH production, up-regulation of these transporters assists in transporting CO_2 into the cell to satisfy the increased demand for Ci under HL.

However, these genes were only slightly affected in my high light experiments discussed earlier in the chapter. A careful analysis of the growth conditions of Hihara et al revealed that the cultures were bubbled with air supplemented with 1% CO_2 and the media was buffered with 20mM Hepes-NaOH, pH 7.0 and the light conditions of the experiment were similar to those I employed. I suspect that levels of Ci under their growth conditions are not likely to be limiting under low light conditions, but the levels of Ci would be at threshold of limitation under HL. It seems that induction of these high affinity inorganic carbon transporters in HL is an indirect affect and it depends on the prevailing Ci conditions.

The results of my HL experiment with cells grown in supplemented Ci (3% CO_2 enrichment of bubbling air) that these high affinity transporters were induced to very low levels. But when the cells were grown in air the transcript abundance of the high affinity transporters were elevated to high levels due to decreased carbon availability. This is because there is an increased flux of reductants and inorganic carbon in the Calvin-Basham-Bennson (CBB) cycle is used to absorb additional reductant generated by HL. The inorganic carbon is used to maintain the redox state of the cell through the CBB cycle. Under HL an increase in reduction potential causes an increase in the inorganic carbon fixation. So during Ci limiting condition these genes encoding for high affinity transporters tend to be active so as to bring in the inorganic carbon in to cell there by maintaining the Ci concentration with in the cell of 30mM (Badger and Andrews, 1982). Under Ci limiting condition the high affinity inorganic carbon uptake transporters are active (Badger et al., 2006). In the HL response under moderate inorganic carbon, the

transcript levels of these high affinity inorganic carbon uptake transporters were up-regulated and the log_2 differential expression ratios are provided in the table 3.16

		Log_2 Expression ratios	
ORF	**Product**	**Air + WTHL15min**	**Air + WTHL30min**
slr1512	***sbtA***	**4.20**	**4.65**
slr1513	***sbtAB***	**1.74**	**3.89**
sll1735	***hypothetical protein***	**2.87**	**3.35**
sll1734	***cupA***	**3.98**	**4.72**
sll1733	***ndhD3***	**3.89**	**4.64**
sll1732	***ndhF3***	**3.32**	**4.77**

Table 3.16: The log_2 differential expression ratios for high affinity inorganic carbon uptake transporters in HL response under moderate Ci condition.

Differences in the coordinate regulation of high affinity Ci transport genes in high light response between the two growth conditions

Interestingly under low inorganic carbon condition resulting simply from a downshift in CO_2 concentration, *cmp*, *cupA*, *sbtA* are coordinately up-regulated (Wang et al., 2004). But when cultures experience inorganic carbon deprivation created by growing under moderate levels of inorganic carbon and exposed to high light this coordinate regulation is not observed, the *cupA* and *sbtA* are up-regulated but not *cmp*. The latter conditions correspond to deficient inorganic carbon that results during increased photosynthesis that pushes the demand for inorganic carbon over the threshold of availability. Why is the expression of members of this functional group of genes no longer coordinately regulated? The explanation for this phenomenon is probably complex. The concentration of inorganic carbon or one of its early uptake products is thought to control the DNA binding activity of LysR-type transcriptional regulators which exert control over these genes (Badger et al., 2006). Perhaps this indicates that light induces a signal that overrides a part of this coordinate induction via these

transcriptional regulators such that the regulator activating the *cmp* operon is modified whereas the LysR regulator controlling *sbtA*, *cupA* continues functioning un-modified. From a biological perspective, this may be important since the high affinity inorganic uptake system encoded by *sbtA*, *cupA* utilizes NADPH to power its activity, whereas the Cmp system is an ABC-type transporter that utilizes ATP energy. Under high light conditions the build-up of NADPH contributes to photoinhibition and oxidative stress hence powering inorganic carbon uptake mainly by the *cupA* system will not only promote photosynthesis but will also lower light-induced damage.

CHAPTER V

MICROARRAY ANALYSIS OF *SYNECHOCYSTIS* SP. PCC6803 TREATED WITH DTT

I. Introduction

The regulatory mechanisms that are involved in modulating gene expression in photosynthetic cyanobacteria remains to be clarified. A considerable body of evidence points to the role of cellular redox state as part of the signaling pathway, although a specific mechanism has not been experimentally determined (El Bissati and Kirilovsky, 2001; Hihara et al., 2003). A number of experiments have used specific photosynthetic electron transport inhibitors to modulate the redox poise of the cell (El Bissati and Kirilovsky, 2001; Hihara et al., 2003; Imamura et al., 2003a). Since regulation of the thiol state of the Calvin cycle enzymes is observed (Lindahl and Florencio, 2003), I wished to begin to test the hypothesis that thiol modifying reagents might also affect gene expression. A significant precedent for manipulation of thiol redox state in cytoplasm has been well-established by the Beckwith lab (Prinz et al., 1997; Katzen and Beckwith, 2000). In these experiments it was found that *Escherichia coli* mutants lacking *trxB* (gene encoding thioredoxin) and glutaredoxin system were unable to grow aerobically, but that these mutants could be rescued by the external addition of DTT. This observation, together with molecular genetic assays probing the thiol state of proteins in the cytoplasm, led to the conclusion that these mutants were unable to maintain protein thiols in a reduced condition (Prinz et al., 1997).

The ability of externally added DTT to reverse this condition, rescuing growth in the process, was interpreted to indicate the activity of a cytoplasmic membrane shuttle that transfers the thiol reductants to the interior of the cells. In the case of the enzymes of the photosynthetic Calvin cycle, catalytic activity is controlled by reduction of regulatory disulphide bridges on the enzymes thereby exposing the active site of the enzyme and thereby switching on the inorganic carbon fixation by the Calvin cycle.

The ferredoxin:thioredoxin system in the chloroplasts is a well studied system. The activity of the photosynthetic electron transport chain produces reduced ferredoxin, that, in turn, reduces $NADP^+$ to NADPH via Fd/NADP oxidoreductase. The reduced NADPH is used to drive reductive metabolism, which is mainly the conversion of CO_2 to sugars. To provide a regulatory connection between these reductive reaction, the reduced ferridoxin can also donate electrons to ferredoxin:thioredox reductase enzyme. This leads to the reduction of the disulphide bridges of different thioredoxin isoforms (photosynthetic organisms typically have several types of thioredoxin). In their reduced form, thioredoxins may reduce the disulphide bridges of the target enzymes in the Calvin cycle as noted above. Thioredoxins thus play an important role in intergating the activity of the photosynthetic electron transport chain and the activity of the Calvin cycle enzymes that utilize the products of the chain, namely ATP and NADPH (Lindahl and Florencio, 2003).

DTT is a chemical analog of cellular thiol reductants such as thioredoxin and glutathinone and it is also capable of affecting the redox state of the cells. I wanted to understand the possible gene targets for this reductant since there is evidence that photosynthesis genes, including those comprising the genes involved in PSII repair, are

controlled by the redox state of cells. The results of this experiment are interesting and consistent with changes in the gene expression occurring due to redox stress, but they are counter intuitive and will require additional experiments to understand the signal transduction pathway involved in thiol redox stress. The results indicate that with the addition of DTT a small subset of genes were affected, indicating DTT had a concerted and a specific effect on gene expression.

II. Results and Discussion

Microarray analysis of DTT treatment in the WT

The RNA isolated at low light was considered as the "reference" and RNA extracted after 15 minutes with the addition of 5 mM DTT was considered as the "treatment". The RNA extraction was conducted as per the protocol discussed in materials and methods.

The WTLL was used as the reference and WT with 5mM DTT was the treatment. There were 158 ORFs which exhibited expression changes of two fold or greater ($\log_2 < -1$ or $\log_2 > 1$). Supervised clustering was performed as described earlier chapter. The ORF were classified into the following clusters based on their expression pattern and their function. While previous microarray experiments exploring redox control in *Synechocystis* sp. PCC6803 have been performed, these experiments employed DCMU and DBMIB, which are specific inhibitors of the electron transport chain.

Functional Cluster –I (High light inducible polypeptides)

The ORFs belonging to this cluster includes the *hli* genes. Interestingly, the *hli* transcripts were highly up-regulated in this treatment. The cells were not subjected to HL and it was thus exciting to observe that addition of DTT to the cells mimicked the effect normally associated with HL stress. Earlier microarray results using redox inhibitors like

DBMIB have also shown that *hli* transcripts were accumulated with addition of these inhibitors. Therefore *hli* genes not only respond to HL but also to change in redox state. Addition of DBMIB prevents the electron flow from PQ (plastoquinone) to cytochrome b_6f complex thereby keeping the PQ pool reduced. The reduced state of PQ may cause the induction of the *hli* genes (Hihara et al., 2003). DTT may induce a thiol redox stress but the pathway affected by DTT is not completely understood. The results indicate that DTT induces a redox stress which can be observed with the up-regulation of all the *hli* transcripts. The transcripts of *hliA* was induced to 24.7-fold, transcripts of *hliB* was induced to 7.6-fold and transcripts of *hliC* was induced to 6.9-fold. High transcript abundance of these high light inducible polypeptides was also observed during low inorganic carbon and nutrient stress conditions (He et al., 2001; Wang et al., 2004). The results suggest that the expression of these genes may be controlled by the redox state of the cell. The ORFs *slr1544, sll1911, sll1483* clustered with *hli* genes hence were included in this cluster. The transcripts of *slr1544*, *sll1911*, *sll1483* also accumulated as the *hli* genes during this treatment. These genes were also induced during the high light treatment and they clustered with the *hli* genes when the hierarchical clustering was performed. The relative fold changes for the *hli* genes and genes that cluster with the *hli* genes are provided in the table 4.1

ORF	***Product***	***Fold change***
slr1544	***unknown***	**4.2**
sll1911	***hypothetical***	**6.5**
sll1483	***hypothetical***	**18.0**
ssl2542	***hliA***	**24.8**
ssr2595	***hliB***	**7.6**
ssl1633	***hliC***	**7.0**
ssr1789	***hliD***	**2.6**

Table 4.1: Relative fold changes in the hli-related genes after the treatment with DTT. The *hli* and the unknown ORFs were up-regulated as observed in the HL treatment.

Functional Cluster-II (Chorophyll and Heme biosynthesis genes)

The data indicates a down-regulation of genes that are required for the synthesis of pigment proteins such as chlorophyll and heme (table 4.2). The genes *chlB*, *chlN* encode for light-independent protochlorophyllide reductase subunits and *chlL* encodes for light-independent protochlorophyllide reductase iron protein subunit. These genes code for enzymes that are involved in the biosynthesis of chlorophyll. The HL data showed that the transcripts levels of these genes are also down-regulated. These genes were also down-regulated in the presence of inhibitors such as DCMU and DBMIB (Hihara et al., 2003). A similar pattern of expression was also observed with addition of hydrogen peroxide which causes oxidative stress (Li and Sherman, 2000). The data also shows that transcript levels of *ho1* are down-regulated with this treatment (table 4.2). Similar results were observed with the addition of hydrogen peroxide (Li and Sherman, 2000). This indicates addition of DTT induces a thiol redox stress similar to oxidative stress as observed with addition of hydrogen peroxide.

ORF	*Product*	*Fold change*
slr0772	***chlB***	**-11.1**
slr0749	***chlL***	**-3.2**
slr0750	***chlN***	**-2.3**
sll1091	***chlP***	**-6.8**
sll1185	***hemF***	**-3.1**
sll1184	***ho1***	**-5.3**

Table 4.2: The relative fold change in the chlorophyll and heme biosynthesis genes after the treatment with DTT. The genes are exhibiting a down-regulation as seen in HL and redox stress. The negative sign indicates down-regulation of the genes in this cluster.

Functional Cluster-III (Iron transport and uptake genes)

Apart from general redox responses, there were also a group of genes which encoded for iron transport proteins, iron uptake proteins and transcriptional regulator belonging to the Fur (ferric uptake regulator) family being up-regulated to high levels

(Table 4.3). Fur acts as a repressor, in the presence of iron it binds to the iron boxes in the target genes and represses their expression. The genes that the Fur repressor appears to control are the iron transport genes and iron uptake genes. There is prevalence in the literature that illustrates, presence of DTT increases the binding affinity of the Fur repressor to the promoter regions of the genes that it regulates. This has been shown by *in vitro* assays using the promoter regions of *myc* operon from *Microcystis aeruginosa* (Martin-Luna et al., 2006). However, in direct opposition to this, the data (table 4.3) shows up-regulation of the small subset of iron uptake and iron transporter genes and also an up-regulation of Fur transcription regulators. This is consistent with the hypothesis that the DTT treatment may actually induce the oxidation, rather than reduction, of celluar thiols. Alternatively, redox stress is created by the DTT and the corresponding regulatory signals dominate the response to the DTT as discussed later.

The ORFs *slr1316, slr1317*, *slr1318* and *slr1319* are physically clustered in the chromosome may function as an operon, since these genes are induced in this treatment. Additionally, the transcript levels of *isiA* and *isiB* are also up-regulated, which is consistent with the fact that these genes are physically clustered in the genome and are expressed as an *isiAB* operon (Singh et al., 2004). Increased transcript abundance of *isiA* is also observed in oxidative stress (Singh et al., 2004). The gene *isiA* encodes for iron stress binding protein and *isiB* encodes for flavodoxin. Its has been shown that IsiB is co-transcribed with IsiA in *Synechoccocus* sp. PCC7942, *Synechococcus* sp. PCC7002 and *Synechocystis* sp. PCC 6803 (Singh et al., 2004). The *isiA* gene was also induced under low iron as well as in the presence of hydrogen peroxide (Singh et al., 2004). The reason for induction of these genes in the presence of DTT is not completely understood. As

with the iron genes, the *isiAB* operon contains a Fur repressor binding site and is presumably affected by the same mechanism proposed above.

ORF	***Product***	***Fold change***
sll1878	***iron(III)-transport ATP-binding protein***	**5.4**
slr1318	***iron-uptake system ATP-binding protein***	**8.4**
slr1319	***iron-uptake system binding protein***	**2.4**
slr1317	***iron-uptake system permease protein***	**2.6**
sll0247	***isiA***	**8.0**
sll0248	***isiB***	**3.1**
slr1295	***futA1***	**18.6**
slr1392	***feoB***	**8.7**
slr0513	***periplasmic iron-binding protein***	**23.9**
slr1738	***transcription regulator Fur family***	**5.7**

Table 4.3: The relative fold changes in the iron transport and iron related genes. The iron related genes were up-regulated in DTT treatment

Functional Cluster-IV (PSII repair regulon)

The hypothesized PSII repair consists of *sigD*, *psbA2*, *psbA3*, *ftsH* (*slr0228*), *ftsH* (*slr1604*) (data provided in table 4.4). The results from the HL data (table 3.10) and evidences from the literature indicate that these genes are regulated by redox stress. The effect observed with the addition of DTT is thiol redox effect. Surprisingly the heat shock proteins or chaperonins do not seem to be up-regulated as observed in the HL data. Since the *ftsHs* and the *psbA* genes were induced, this could be attributed to the fact that D1 is being damaged and repaired. The *psbA2* and *psbA3* genes are induced two to three-fold whereas *sigD*, *ftsH* (*slr0228*), *ftsH*(*slr1604*) are induced four to five- fold. The *gro EL-2* was not induced in this experiment with DTT but prolonged exposure to DTT could induce the heat shock proteins and molecular chaperonones.

ORF	*Product*	*Fold Change*
sll2012	***sigD***	**5.7**
slr0228	***ftsH***	**5.0**
slr1604	***ftsH***	**4.6**
slr1311	***psbA2***	**3.3**
sll1867	***psbA3***	**2.4**
sll0416	***groEL-2***	**0.6**

Table 4.4: Relative fold change in gene expression in the PSII repair regulon.

III. Conclusion

As observed from the results there was a small subset of genes that exhibited changes in gene expression with the addition of DTT. DTT is biochemical analog of thioredoxin and is known to change the thiol redox state of the cell. Additional experiments are needed to pursue these intriguing, but still preliminary results. The results suggest that DTT may, in contrast to the *E. coli* work (Prinz et al., 1997; Katzen and Beckwith, 2000), be causing an oxidizing effect rather than a reducing effect within the cells. This is consistent with the observation that *AhpC*, which codes for Alkyl hydroperoxide reductase and it belongs to the family of thiol specific antioxidant, was up-regulated to 16.5-fold that provides an independent conclusion that DTT is causing an oxidizing effect within the cells. Interestingly, other redox stress enzymes such as catalase and superoxide dismutase, were not up-regulated by the DTT treatment. Therefore, if DTT is inducing a redox stress, it is likely to be a very specific thiol stress. As stated earlier by work done in Beckwith lab, there is a shuttle system that transports the reductants from outside to the interior of the cells in *E.coli*. There could a similar system occurring in cyanobacteria as well. The reason for iron related genes being up-regulated is not completely understood. In the context of the subject of my thesis, the most interesting result is the up-regularion of the PSII repair regulon. At this stage we

cannot discriminate between the possibilities of up-regulation being due to photoinhibitory damage caused by thiol redox stress or if the transcriptional control proteins are more directly controlled by thiol state. Clearly this needs to be explored more thoroughly. Another interesting finding is the down regulation of the chlorophyll biosynthesis genes. One reason for chlorophyll and heme biosynthesis genes being down-regulated could be that the free chlorophylls may be released due to photoinhibitory damage to D1 as seen in the HL effect or that these genes may also be regulated by thiol state of the transcription factors controlling their expression. Whatever be the mechanism, the coordinate up-regulation of the *hli* genes and down-regulation of chlorophyll biosynthesis genes seems to make functional sense. The *hli* genes may be induced to sequester these free chlorophylls, hence play an important role in photoprotection. Obliviously, production of additional chlorophyll under these conditions is wasteful at best and dangerous are worst. Again, whether the expression of *hli* genes and the chlorophyll genes is controlled by the oxidation state of cysteines on the regulatory proteins of *hli* genes and chlorophyll genes remains speculative for the present time. Since the mechanistic basis of these results is not understood, additional experiments using diamide or oxidized DTT, that have opposite effects of DTT could provide a clue to define better the thiol redox effects hinted at by my work.

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VITA

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Light is the primary source of photosynthesis but one of draw backs of high light is the formation of reactive oxygen species. This potentially damages the D1 protein, this proteins forms the core of PSII. DI protein is capable of undergoing rapid turnover in high light and this turnover mechanism involves various factors such as FtsH proteases (*slr0228*) are involved in the removal of damaged D1, transcription regulators, including the sigma factors such as SigD that are involved in the regulation *psbA* genes under high light, and chaperones. The project was aimed to investigate the role of SigD in regulation of the hypothesized PSII repair regulon which includes *psbA2*, *psbA3*, *ftsH*(*slr0228*), *ftsH*(*slr1604*) and *groEL-2*. The findings suggest that *ftsH*(*slr1604*) also plays a role in the repair mechanism. But apart from these set of genes which seem to be coordinately regulated by SigD, there are other genes such as *hli* (high light inducible polypeptides) and genes that involved in the biosynthesis of chlorophyll and heme proteins. All these factors play an important role in the PSII repair mechanism.

ADVISER'S APPROVAL: Dr. Robert L Burnap