Characterization of a DEAD-box RNA helicase mutant Δ*crhR* in *Synechocystis* sp. PCC 6803

A thesis submitted to the University of Hyderabad for the award of Doctor of Philosophy in Plant sciences

By

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DECLARATION

I, Sireesha Kodru, hereby declare that this thesis entitled **"Characterization of a DEAD-box RNA helicase mutant Δ*crhR* in *Synechocystis* sp. PCC 6803"** submitted by me under the guidance and supervision of Dr J.S.S. Prakash is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Characterization of a DEAD-box RNA helicase mutant Δ*crhR* in *Synechocystis* sp. PCC 6803"** is a record of bonafide work done by **Sireesha Kodru**, a research scholar for Ph.D. programme in Plant Sciences, Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Dr. J.S.S. Prakash
(Supervisor)

(Head of the Department) **(Dean of the School)**

Dedicated to my beloved parents

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Abbreviations

A_{max}	absorbance maximum
Amp	ampicillin
APC	allophycocyanin
ATP	adenosine 5.-triphosphate
bp	base pairs
Chl	chlorophyll
Cm	chloramphenicol
CP43 and CP47	photosystem II antenna chlorophyll-protein subunits
D1 and D2	photosystem II core complex protein subunits
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease I
Fm	maximum fluorescence yield of closed PSII RCs in dark-adapted state minimum fluo
Fo	yield of open PSII RCs in dark-adapted state
Fv	maximum fluorescence change in dark-adapted state (Fm-Fo)
HEPES	N-(2-hydroxyethyl)piperazine N.-(2-ethane sulfonic acid)
HL	high light
HLIP	high light inducible proteins
IgG	immunoglobulin G
IPTG	isopropyl ß-D-thiogalactoside
Km	kanamycin
LHC	light-harvesting complex
LL	low light
NADP+	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NADP(H)	nicotinamide – adenine dinucleotide phosphate (reduced form);
NDH-1	type 1 NADPH dehydrogenase;
NPQ	Non-photochemical quenching
OD	optical density
OEC	oxygen-evolving complex
ORF	open reading frame
P680	primary electron donor of PSII reaction center
P700	primary electron donor of PSI reaction center
PBS	phycobilisome
PC	phycocyanin
PE	phycoerythrin
PCR	polymerase chain reaction
PQ	plastoquinone
PQH_2	plastoquinol
PsaA and PsaB	photosystem I core complex protein subunits
PSI	photosystem I
PSII	photosystem II

RC	reaction center
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
Fd ox and Fd red	ferredoxin in oxidized and reduced forms, respectively
Ox	terminal oxidase
PC	plastocyanin
PQ	plastoquinone
SDH	succinate dehydrogenase.

Introduction

Chapter 1

1. Introduction

1.1. Abiotic stress response

Stress on living cells can be defined as those environmental conditions capable of causing potentially injurious effects. Clearly if the magnitude and/or duration of the unfavourable changes are sufficient, then a certain degree of strain can be developed in the organism. Then the organism is said to be under stress. Living organisms have innate strategies by virtue of which they are capable of acclimatizing to stress (unfavourable changes) by inducing and/or optimizing several molecular mechanisms there by able to survive in the changed environment. Responses of living organisms to a stress involve many biological macromolecules, including proteins and lipids during acclimation to a changed environment. Studies of the responses of the microbes and plants to environmental stress (abiotic stress) such as salinity, drought, heat, cold etc., form a thrust area of contemporary biological research. Compared to the microorganism, plants are rather difficult to study and the bulk information we have today on stress responses of plants to abiotic streses generally arose from the studies of microbial systems such as *Escherichia coli*, *Bacillus* and the cyanobacterium *Synechocystis* sp. PCC 6803(here after *Synechocystis*). In *Synechocystis*, abiotic stress signal is sensed and transduced to the target genes by two component signal transduction systems (Fig.1.1). The stress specific genes thus upregulated leads to synthesis of proteins involved in operation of mechanisms for acclimation to stress condition (Fig.1.1).

Temperature is an important environmental factor influencing survival of microorganisms. Adaptation to temperature fluctuation is essential for the survival of all living organisms. An increase in temperature results in a specific heat shock response, which is shared by all organisms from bacteria to higher eukaryotes. The heat shock response is characterized by the synthesis of a set of highly conserved set of heat inducible proteins (Strauch et al., 1989; Lipinska et al., 1990; Spiess et al., 1999). Similarly, in many of

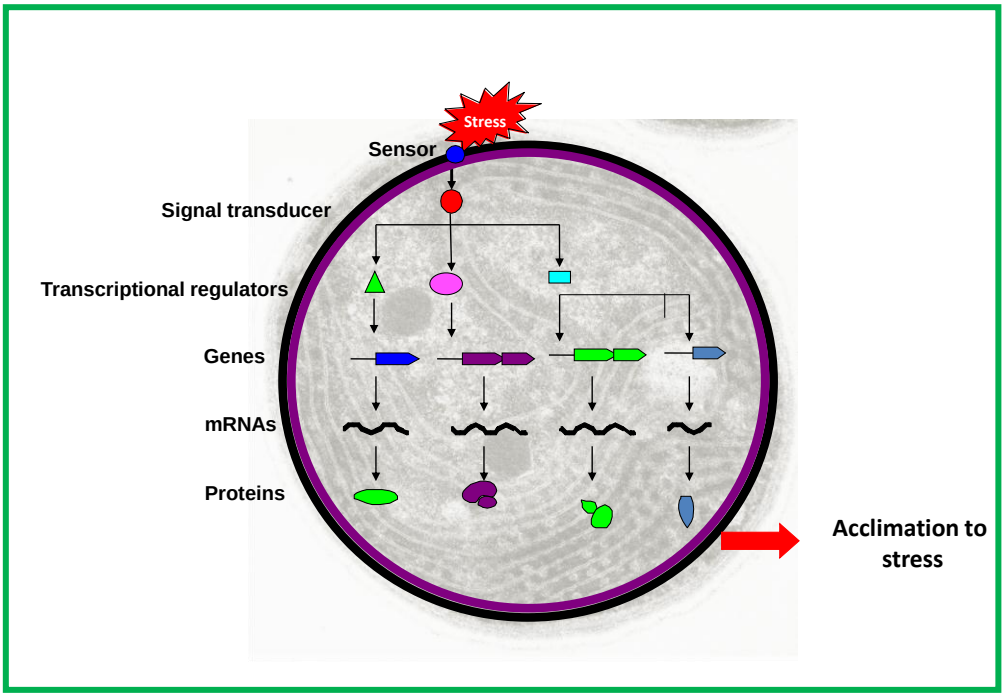

Fig.1.1. A general scheme showing the responses of a cyanobacterial cell to environmental stress. Stress signal is sensed by histidine kinases (sensors) located either in the membrane or cytoplasm. Activated sensory kinase transduce signal to the target genes via response regulators (signal transducers) by phosphorylation. Genes thus upregulated leads to induced molecular level responses, thereby cell acclimatize to stress condition.

bacterial species there are common survival mechanisms which are operative for adaptation in response to chilling temperature. One of such survival mechanism is to maintain their adequate membrane function, which is achieved by regulating the fluidity/physical state of their membranes. Without regulation, an organism shifted from a high to low temperatures would have membrane lipids with suboptimal fluidity, resulting in subnormal membrane function. When poikilothermic organisms such as bacteria, fungi, protozoa, plants and animals are exposed to temperatures below those of their normal growth conditions, increases occur in the degree of unsaturation of the fatty acids in membrane lipids (Los and Murata, 1999). Besides the temperature dependent membrane fluidity optimization, several other molecular mechanisms are responsible for acclimation of living organisms to low temperature stress.

1.2. Cold-shock response

Down shift of temperature lead to several physico-chemical changes that severely affects cellular functions in bacteria. Immediate cellular response to the changes is to resist the life-endangering influence of low temperature. The sum of all cellular reactions to overcome the changes associated with drop in temperature is called 'cold shock' response. The 'cold shock' response in microorganisms is a transient phenomenon that affects growth rate of cell, membrane structure and function, and rates of DNA, RNA, and protein synthesis.

1.3. Mechanisms of cold adaptation

1.3.1. Changes in lipids and membrane properties

One of the most well characterized changes that occur in cold-shocked bacterial cell is the change in physical property of membrane, namely membrane fluidity. With the drop in temperature, the cell membrane changes from a liquid crystalline state (disorder or fluid) to gel phase (ordered or rigid). Normally, an optimum fluidity is required for membrane function, which is regained in bacteria by changing the nature of constituting lipids and fatty

acids (Fig.1.2). The process, called "homeoviscous adaptation"(Sinesky, 1974), is achieved by one or combination of the following changes in phospholipids: (i) increase in the level of unsaturated fatty acids, (ii) reduction in chain length, (iii) increase in methyl branching fatty acids, and (iv) increase in the ratio of anteiso- to isoform of branched chain fatty acids. The biochemical process of introducing these changes may vary among different bacteria (Russel, 1990; Russel and Nichols, 1999). For example, the unsaturation of membrane phospholipids in *E. coli* occurs by the so-called anaerobic pathway, using β-ketoacyl-ACP synthase II (*fabF* gene product) that converts newly synthesized palmitoleic acid (n-16:1 Δ9-*cis*) to *cis*-vaccenic acid (n-18:1 Δ11-*cis*), which is in turn incorporated into *sn*-2 position of phospholipid backbone, while it is achieved by aerobic pathway, using desaturase enzyme that directly introduces double bond into saturated fatty acids. In mesophilic cyanobacteria (*Synechocystis sp.* PCC 6803 and *Synechococcus sp.* PCC 7942), the membrane bound desaturase introduces double bonds into the fatty acids (mainly at Δ12 and Δ15 positions) while these are bound to membrane phospholipids (Los and Murata, 1999). Low temperature increases activity of the desaturase enzyme due to regulation at both transcription and translation level. In mesophilic *Bacillus subtilis* the membrane fluidity following cold-shock is altered both due to increased unsaturation of fatty acids by activity of desaturase (Δ5) and conversion of *iso*-branched to *anteiso*-branched saturated fatty acids. Isoleucine provides the precursors for branched chain fatty acids and regulates the latter activity (Mansilla et al., 2004).

1.3.2. Changes in lipopolysaccharides

Lipopolysaccharides (LPS) are unique component of outer membrane (OM) in the Gram-negative bacteria. These glycolipids are exclusively present in outer leaflet of the asymmetric outer membrane-bilayer, where the inner leaflet is made up of phospholipids, similar to the cytoplasmic membrane. Changes in LPS regulate the properties of OM including

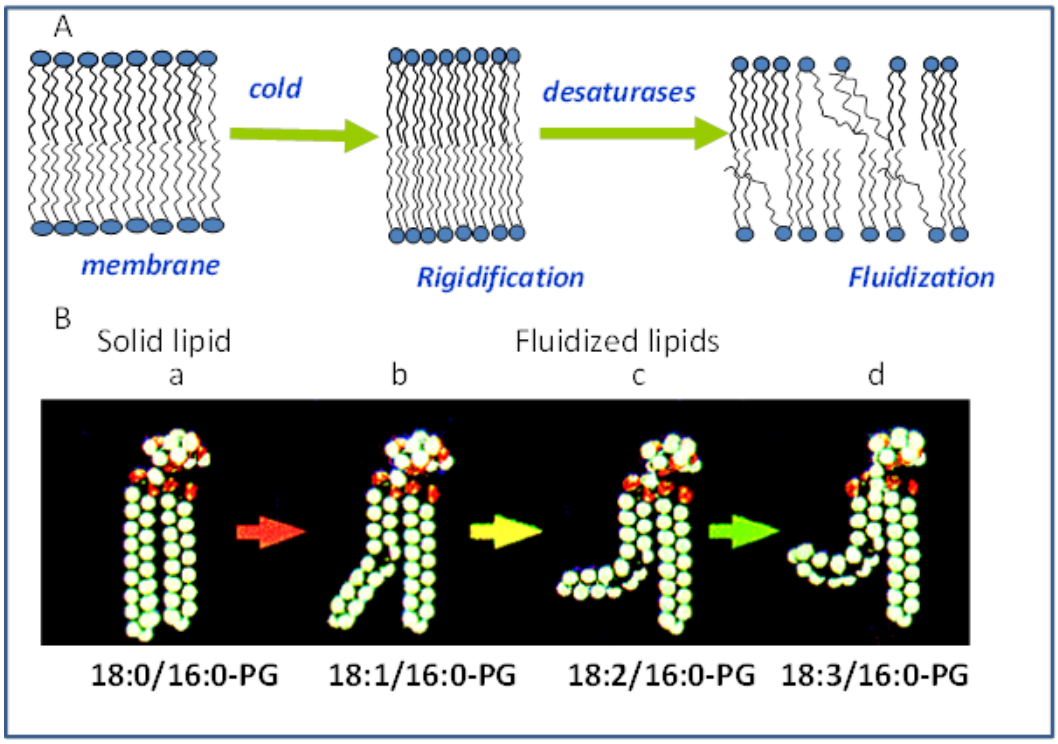

Fig.1.2. Model representing the changes in membrane lipid bilayer at low temperatures. Low temperature leads to membrane rigidification and the membrane would be in the gel phase at low temperature (A). Introduction of double bonds in the lipid fatty acid converts the solid lipids 18:0/16:0-PG to 18:1/16:0- PG and may be finally to 18:3/16:0-PG, thus helping to restore the necessary fluidity at low temperature (B, a to d).

permeability, and possibly the transport process by changing the properties of the embedded porin channels. In *E. coli*, the activity of the palmitoleoyltransferase (LpxP), which incorporates a palmitoleoyl moiety into the lipid A is upregulated at low temperature; therefore it has been speculated that incorporation of the unsaturated palmitoeic acid might alter the physical properties of the outer membrane suitable for cellular activity in cold environment (Carty et al., 1999). It has been reported that the amount of unsaturated fatty acids in the lipid A moiety of LPS, in general, increases upon downshift of temperature (37^{o}C to 15^{o}C) in many mesophilic bacterial species, which might be responsible for maintaining a homeoviscosity of the outer membrane.

1.3.3. Change in protein profile

Following cold shock, there is a transient inhibition in the synthesis of most cellular proteins, which is manifested by the lack of cell growth. The extent of protein synthesis inhibition varies in different bacteria. In *E. coli*, there is a total inhibition of housekeeping protein synthesis, which is accompanied by transient synthesis of a new set of proteins, which presumably help the cells not only to protect them from cold-induced injury but also help to resume growth by adjusting cellular machinery to slower metabolic rate under low temperature. At least 15 proteins are induced in *E. coli* during the first hour of cold-shock, which are repressed soon to basal level (Thieringer et al., 1998; Yamanaka, 1999). In *B. subtilis* the protein synthesis inhibition is only partial and at least 75 proteins are synthesized following the cold shock (Graumann et al., 1996; Hebraud and Potier, 1999). Two kinds of proteins are mainly synthesized during the growth lag following down shift of temperature: so-called 'cold-shock' proteins (CSPs) or class I protein, whose level increases sharply for short period within the lag phase, and the cold-acclimation proteins (CAPs) or class II protein, the level of which increases gradually to a moderate level and does not fall as fast as CSPs. Examples of the class I protein include cold shock proteins CspA, CspB, CspG, CspI,

RNA helicase CsdA, ribosome binding protein RbfA, transcription factor NusA, and exoribonuclease PNPase while the class II includes histone-like protein H-NS, recombination protein RecA, DNA gyrase subunit A, translation initiation factor IF2α, trigger factor (TF), pyruvate dehydrogenase subunit E1, and dihydrolipoamide dehydrogenase (Jones et al., 1987; Thieringer et al., 1998; Yamanaka, 1999). Thus, there is a distinct change in protein profiles of bacteria during acclimatization to lower temperature of growth. Following a growth lag, the cold-shocked cells resumecell division, transcription and translation of housekeeping genes, and continue the synthesis of "Cold acclimative proteins" (CAPs) for some more time before reaching to the steady state level of growth. The growth rate at low temperature is slower, and hence all the metabolic activities are adjusted to the new rate.

Proteins of diverse type are involved in diverged cellular functions during cold acclimation. Among them, the CspA family of proteins constitute the most common type of 'cold shock' proteins in bacterial species. They are small acidic proteins with molecular mass ~7.0 kDa. The first identified member of the family is CspA from *E. coli*, whose homologues were subsequently identified from several bacteria (Graumann et al., 1996; Hebraud and Potier, 1999; Thieringer et al., 1998). Cold shock proteins are homologues, in fact, are the most conspicuous group of protein at low temperature in any bacteria, which could constitute more than 10% of total cellular proteins in *E. coli* upon cold-shock. Among the nine homologues, only four (CspA, CspB, CspG and CspI) are cold inducible in *E. coli* (Yamanaka, 1999; Phadtare and Inouye, 2004). In Gram-positive bacterium *B. subtilis*, CspB of the three homologues (CspB, CspC, and CspD) is cold inducible (Graumann et al., 1996; Kaan et al., 2002). This suggests that there is a functional redundancy, as well as probable division of labour among the members of the group. In fact, *E. coli* with only quadruple deletion but not double or triple deletions of *cspA*, *cspB*, *cspG*, and *cspI* genes are cold sensitive (Phadtare and Inouye, 2004).

1.3.4. Translation machinery and protein synthetic capacity at low temperature

Immediate effect on bacterial cells upon shift to low temperature is dramatically manifested by inhibition of protein synthesis, presumably due to blockage at the initiation phase of translation and stabilization of secondary structures of mRNA. Inhibition of translation is reflected by accumulation of 70S monosomes and concomitant decrease in polysomes. RbfA, a 30S ribosomal subunit associated protein is thought to play a crucial role in relieving the translational block at low temperature in *E. coli* (Fig.1.3). RbfA is a cold inducible protein and its deficiency leads to cold sensitive phenotype (Jones and Inouye, 1996). The stabilization of mRNA secondary structure, a possible cause for hindrance of translation initiation and pausing of elongating polysome on mRNA are probably overcome by CspA group of RNA chaperone proteins (Fig.1.3). It has been proposed that CspA binds to nascent mRNA during transcription and thereby prevents the formation of intramolecular hydrogen bonds of RNA chain, and thus facilitates coupling of transcription to translation. The 'DEAD box' RNA helicase, like CsdA probably also play a crucial role by actively unwinding duplex structures of RNA at the expense of ATP. Deletion of *csdA* impairs growth of *E. coli* at 15^{o}C (Jones et al., 1996). Although a recent study shows the association of CsdA protein to RNA degrading machinery of the bacterium, the possibility remains that CsdA might also play a role in destabilizing RNA helices for facilitating translation at low temperature.

1.3.5. Transcription and RNA polymerase

Upon cold shock, *E. coli* exhibits transient inhibition of transcription of housekeeping genes, while the cold-shock genes are induced. For example, DesR was found to be responsible for up regulation of *des* gene that encodes desaturase in *B. subtilis* (Aguilar et al., 2001). Similarly, a 'response regulator' protein (RreI) of temperature sensing two-component regulatory system (Hik33, Hik19/RreI) was found to be responsible for upregulation of *des* genes in *Synechocystis* PCC 6803 (Suzuki et al., 2001). CspA protein was originally reported

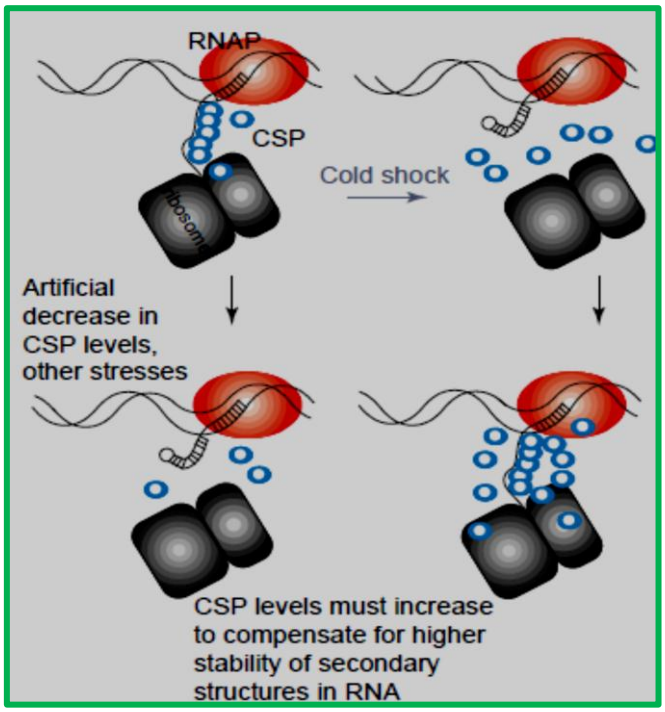

Fig:1.3. Model for the function of cold shock proteins (CSPs) as RNA –chaperones that couple transcription and translation of mRNA. The stabilization of mRNA secondary structure, a possible cause for hindrance of translation initiation and pausing of elongating polysome on mRNA are probably overcome by CspA group of RNA chaperone proteins. Csp binds to nascent mRNA during transcription and thereby prevents the formation of intramolecular hydrogen bonds of RNA chain, and thus facilitates coupling of transcription to translation. Adapted from Graumann and Marahiel, 1998.

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to bind the promoter region of cold inducible genes *gyrA* and *hns* in *E. coli* (Thieringer et al., 1998). Transcriptional antitermination factor, such as *nusA*, might also play a role in transcription at low temperature. It is generally believed, without any strong evidence, that the de repression, rather than a positive activation, might be responsible for low temperature induced gene expression in bacteria.

1.3.6. Proteins associated with RNA stability and RNA degradation

It is now believed that RNA stability plays a major role in increasing the level of cold induced mRNAs at low temperature. For example, *cspA* mRNA although transcribed at 37^{o}C is not detectable at this temperature due to quick degradation, whereas at 15^{o}C, the level of *cspA* mRNA increases several fold (>10 folds) due to increased half-life (stability) of the mRNA (Goldenberg et al., 2003). Similarly, RNA stability was found to play a major role in the induction of des genes (*desA*, *desB*, and *desD*) at low temperature in the cyanobacterium *Synechocystis sp.* PCC 6803 (Los et al., 1997). It has been noticed that the low temperature inducible genes generally contain a 100 to 200 base long 5'-untranslated region (5'-UTR), which presumably plays a critical role in the increased stability of mRNA in addition to efficient translation at low temperature (Yamanaka et al., 1999). Interestingly, RNA degrading machinery (degradosome) has recently been found to undergo compositional changes at low temperature. In *E. coli* under normal growth conditions, RNA degradosome is comprised of the endoribonuclease RNaseE, exoribonuclease PNPase, RNA helicase RhlB and the carbohydrate metabolizing enzyme enolase (Py et al., 1996; Rauhut and Klug, 1999). At low temperature, the RNA helicase CsdA probably becomes a component of degradosome by replacing RhlB (Prud'homme-Genereux et al., 2004). In the psychrotrophic bacterium *Pseudomonas syringae*, the RNA degradosome has RNaseE complexed with exoribonuclease RNaseR and the RNA helicase RhlE (Purusharth et al., 2005). It is now believed that recruitment of different components to the degradosome under different environmental

conditions including low temperature might modulate the RNA degrading machinery leading to altered half-life of different class of mRNAs in cell. In this context, it is to be noted that PNPase is a cold inducible protein, and *pnp* gene inactivation leads to cold sensitive phenotype in mesophilic *E. coli*, and psychrotrophic *Yersinia enterocolitica* (Goverde et al., 1998).

1.3.7. Protein folding and protein degradation

Cytoplasm is protected against the perils of protein misfolding by two mechanisms: molecular chaperones which facilitate proper folding and the protein degradation machinery, which degrades misfolded proteins. Low temperature causes denaturation and aggregation of proteins. Studies suggest that proper folding of proteins and/or maintenance of quarternary structure of proteins at low temperature might be essential for cold adaptation process (Strocchi et al., 2006). Hsc66, a member of the Hsp70 class of molecular chaperones is cold inducible, and was proposed to act as cold shock molecular chaperone in *E. coli* (Lelivelt and Kawula, 1995). The importance of chaperone function in proper folding of proteins and promoting growth at low temperature was demonstrated by the ability of the GroEL and GroES homologues (Cpn60 and Cpn10) from the Antarctic bacterium *Oleispira antarctica* strain RB-8T in supporting growth of *E. coli* at 4^{o}C (Ferrer et al., 2003). Trehalose, which has been proposed to function like a chemical chaperone, was also found to protect *E. coli* cells at 4^{o}C. A mutant of *E. coli* that was unable to produce trehalose died much faster than the wild-type at low temperature (4^{o}C) (Kandror et al., 2002). The cold inducible trigger factor (TF), was proposed to play an important role in cold adaptation. This protein has multiple functions such as peptidyl-prolyl-*cis-trans* isomerisation (PPIase), binding to nascent polypeptides on ribosomes, ability to bind GroEL and affinity to unfolded proteins, promoting degradation of certain polypeptides during acclimation to low temperature. Inactivation of the gene for TF resulted in decreased viability of *E. coli* at cold (Kandror and

Goldberg, 1997). In the psychrotrophic bacterium *Shewanella sp*. S1B1, a homologue of FKBP family of proteins having PPIase activity has been experimentally shown to play a role in the cold adaptation (Suzuki et al., 2004). Two caseinolytic proteases (ClpB and ClpP) of *Synechococcus sp*. 7942 were also found essential for low temperature growth (Porankiewiz et al., 1998). Thus, protein folding and protein degradation also plays a major role in the cold adaptation process.

1.3.8. DNA Supercoiling is involved in the perception of stress signals and regulation of gene expression

Alterations in the supercoiling of genomic DNA play an important role in the regulation of gene expression in response to environmental stress in both Gram-negative and Gram-positive bacteria (Wang et al., 1993; Dorman, 2006). It has been proposed that temperature-dependent alterations in DNA supercoiling might be one of the sensory mechanisms that regulate the expression of genes involved in the acclimation to low temperature (Prakash et al., 2009; Los, 2004). Salt stress and hyperosmotic stress also affect the negative supercoiling of DNA and regulate gene transcription (Cheung et al., 2003). Studies of changes in the supercoiling of DNA were initially limited to plasmid DNAs in *E. coli*, *B. subtilis* and *Salmonella typhimurium*. Therefore, changes in gene expression due to changes in the supercoiling of chromosomal DNA, have mainly been assumed on the basis of changes in the linking numbers of plasmids (Adamcik et al., 2002; Aoyama and Takanami 1988; Franco and Drlica, 1989). An inhibitor of DNA gyrase, novobiocin has been used to examine the effects of changes in the negative supercoiling of DNA on the genome-wide expression of genes in *Synechocystis* in response to cold stress (Gellert et al., 1976; Los, 2004). Novobiocin interacts with the ATP-binding site of the β-subunit of DNA gyrase. Cold stress causes an increase in the negative supercoiling of the promoter region of the *desB* gene for a fatty acid desaturase and directly controlled its expression at low temperatures (Los, 2004;

Prakash et al., 2009), pointing that temperature-induced changes in supercoiling of DNA might contribute to stress-induced gene expression in cyanobacteria. DNA microarray-based analysis of gene expression demonstrated that novobiocin, which inhibits stress-induced changes in DNA supercoiling, regulated the transcription of many genes that are involved in stress responses including the genes that are obligatory for acclimatization of cells to changed environments. The function of the two-component regulatory systems, which are known as sensors and transducers of salt, cold, and heat stress (in particular, Hik33 and Hik34 histidine kinases), depends on the degree of supercoiling of the genomic DNA (Prakash et al., 2009). Thus, DNA supercoiling might regulate transcription of stress-inducible genes directly, and/or provide a permissive background for regulatory proteins, which switch on or off the expression of the downstream genes and ensure successful acclimatization of cells to stress conditions.

During perception of the cold stress signal, the sensory histidine kinase Hik33 feels rigidification of the membrane. Hik33 controls most of the highly cold-inducible genes, and the activation mechanism for other cold-inducible genes remained unclear. The cluster analysis of the results obtained with DNA microarrays revealed that expression of the majority of the cold-inducible genes depend on cold-induced increase in negative supercoiling of the genomic DNA (Fig.1.4). At least, cold-induced transcription of *crhR* for RNA helicase and *rbpA1* for RNA-binding protein could be prevented by the inhibition of the DNA gyrase. It is well known that acclimation to low temperatures require expression of genes for ROS inactivation, for ribosomal proteins (excess of which compensates for a drop in translational speed at low temperatures), for RNA chaperons (to maintain the RNA matrices unwound), for cell wall and lipid metabolism (to protect the destruction of cell walls and to maintain the fluidity of membranes under certain level, which prevents cold-induced lipid phase separation) (Los and Murata, 2004). All these genes necessary for acclimatization

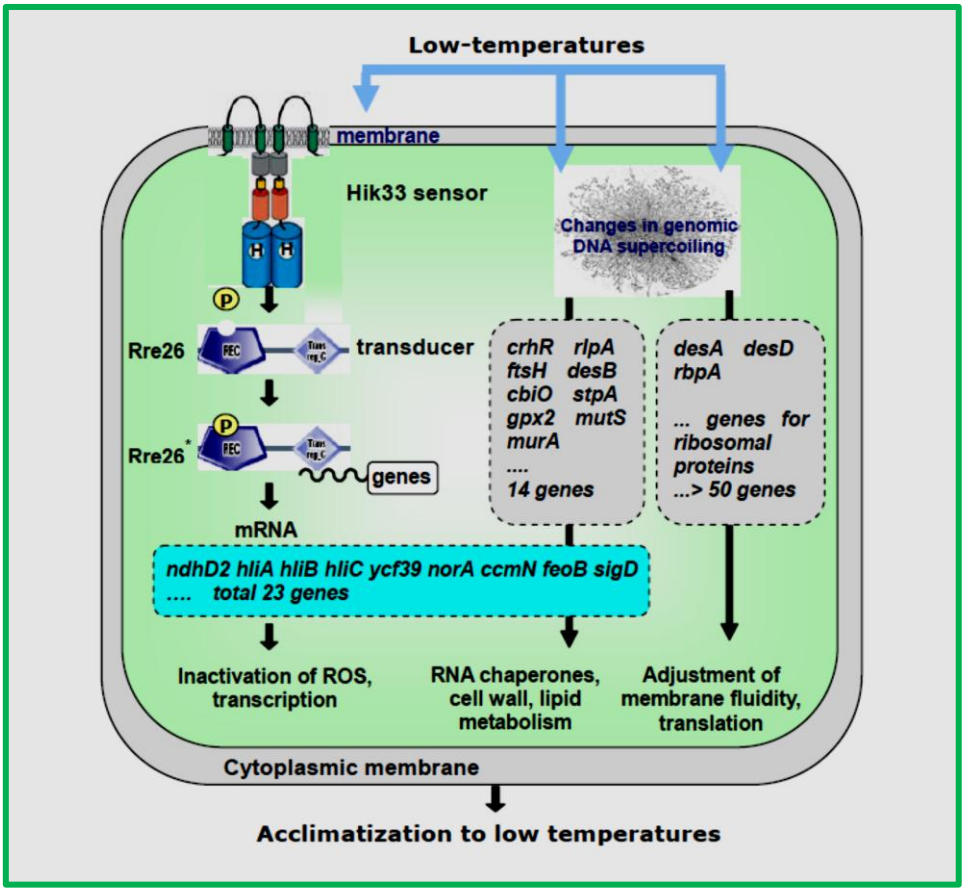


Fig.1.4. A scheme of the perception and transduction of cold-stress signals by cyanobacterial cells. The sensory histidine kinase Hik33 perceives the temperature-induced rigidification in the cytoplasmic membrane. Upon autophosphorylation of the Hik33 dimer, it transduces the phosphoryl group to the response regulator Rre26, which binds to promoter region of the genes to induce their transcription. The Hik33-Rre26 two-component sensor and transduction system regulates a part of cold-inducible genes. This part, however, is also under control of the cold-induced increase in negative supercoiling of the genomic DNA. The latter mechanism controls cold-induced transcription of genes for RNA chaperons, translation, cell wall metabolism, and regulation of the membrane fluidity. Adapted from Los et al., 2008.

to low temperatures are activated during the cold-induced changes in genomic DNA supercoiling.

1.3.9. Cold sensors and cold signal transducers

1.3.9.1. The Hik33-Rre26 system controls the expression of cold-inducible genes

The histidine kinase Hik33 (*sll0698*) has been identified as a cold sensor in *Synechocystis* (Suzuki et al., 2000). Hik 33 has been earlier described as a component of the drug-resistance machinery DspA (Bartsevich and Shestakov,1995;Tu et al.,2004) and it is a homolog of NblS of *Synechococcus*, which may be involved in the regulation of genes that are induced under nitrogen limiting conditions (Waasbergen et al.,2002). However, the Hik33 does not seem to contribute significantly to the transduction of nutrient-related signals in *Synechocystis* (Zabulon et al., 2007). DNA microarray analysis of *hik33* mutant cells indicated that it regulates the expression of 23 of 38 highly cold-inducible genes (Fig.1.5). These 23 genes include *ndhD2, hliA, hliB, hliC, fus, feoB, crtP*, as well as genes for proteins of unknown function. Yet, 15 of the 38 cold-inducible genes were not regulated by Hik33. It was suggested that *Synechocystis* might have another pathway for transduction of the low-temperature signal. The genes that are not controlled by Hik33 include highly cold-inducible genes, e.g., *crhR*, which encodes an RNA helicase and *rbp1*, which encodes a RNA-binding protein.

1.4. Genome-wide analysis of cold-stress inducible genes in *Synechocystis*

1.4.1. Cold-inducible genes in *Synechocystis*

There are several comprehensive reviews of the responses of cyanobacteria to cold stress (Murata and Wada, 1995; Los and Murata, 1999, 2004). Cold-inducible genes have been grouped into several categories, as follows: (1) genes for fatty acid desaturases that are responsible for adjustments in membrane fluidity; (2) genes for RNA-binding proteins (Rbps) that, probably, serve as RNA chaperones similarly to the Csp proteins of *E. coli* and *B.*

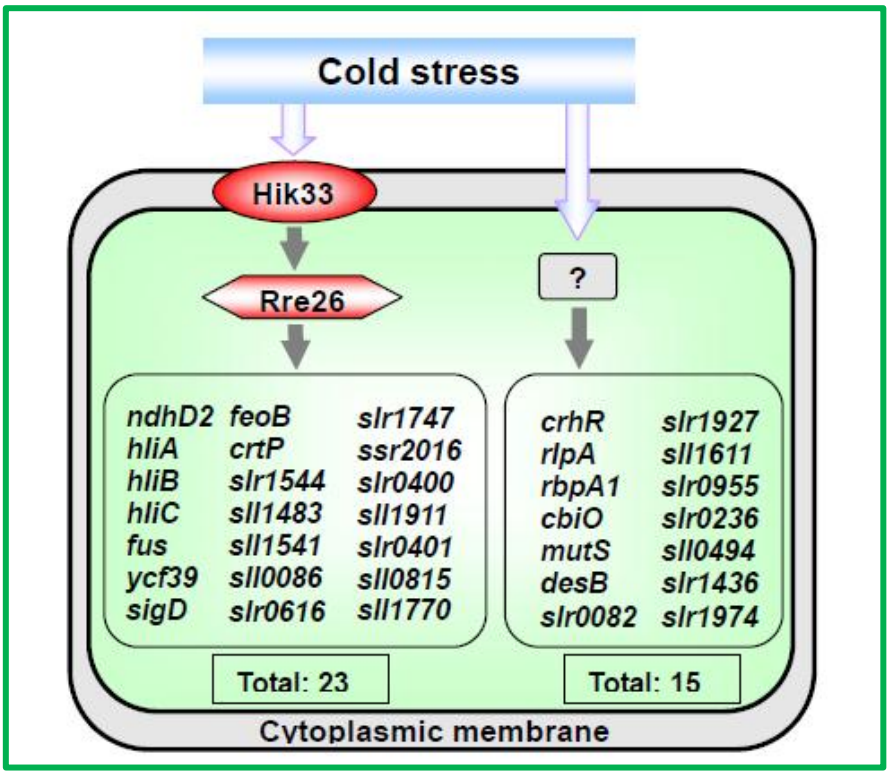


Fig.1.5. Schematic presentation of two-component system Hik33-Rre26 involved in the transduction of low-temperature stress. Primary signals are represented by open blue arrows. The sensory histidine kinase Hik33 supposedly perceives the temperature-induced rigidification in the cytoplasmic membrane. It tranduces the phosphoryl group to the response regulator Rre26, which itself might bind to promoter region of the genes to induce their transcription. Uncharacterized mechanisms are represented by question marks. Genes with induction factors (ratios of transcript levels of stressed cells to those of non-stressed cells) higher than 3**:**1 are included in this scheme. Adapted from Los et al.,2008.

subtilis; (3) genes for RNA helicases that destabilize the secondary structures of mRNAs, thereby overcoming inhibition of the initiation of translation at low temperatures; (4) genes for ribosomal proteins, an excess of which is necessary for acclimation of the translational machinery to cold; (5) genes for caseinolytic proteases that participate in the renewal of photosystem (PSII) and (6) various other genes that do not fall in any of the other five categories. The availability of genome-wide DNA microarrays of *Synechocystis* has provided new opportunities for studies of responses of the entire genome to cold stress (Suzuki et al., 2001). The expression of close to 50 genes is strongly induced in *Synechocystis* under cold stress (Table.1). In addition to the above-mentioned groups, some other important genes appear to be induced at low temperatures, namely, the *rpoA* gene for RNA polymerase; the *sigD* gene for sigma factor D; the *fus* gene for elongation factor EF-G; the *hliA*, *hliB*, and *hliC* genes that encode high light-inducible proteins, which are involved in the regulation of photosynthesis; the *ndhD2* gene for subunit 4 of NADH dehydrogenase; the *cytM* gene for an alternative form of cytochrome *c*; several genes that are expressed in response to oxidative stress; and several genes for proteins of as yet unknown function (Table.1). Thus, it is evident that cold stress enhances the expression of many genes whose products control membrane fluidity, transcription, translation and the energy status of the cell.

The *crhR* gene for RNA helicase is one of the genes whose expression is highly induced upon low-temperature stress in *Synechocystis* (Suzuki et al., 2001; Los et al., 2008). The database of genome sequences available at http://www.kazusa.or.jp suggests that *Synechocystis* contains only one gene, *crhR* (open reading frame, *slr0083*), for RNA helicase. However, the function of this RNA helicase during low temperature acclimation is not known.

ORF	Gene	Encoded protein		Ratio of transcript levels
± 7.1	*slr1291*	*ndhD2*	NADH dehydrogenase I chain M	22.0 :
± 1.0	*ssl2542*	*hliA*	High light-inducible protein	20.7 :
± 4.8	*slr0083*	*crhR*	ATP-dependent RNA helicase	16.3 :
± 3.2	*ssr2595*	*hliB*	High light-inducible protein	10.6 :
0.8	*sll1541*		Putative lignostilbene-dioxygenase	6.4 ±
0.5	*slr1105*	*fus*	Elongation factor EF-G	6.2 ±
1.0	*sll0086*		Arsenical pump-driving ATPase	5.9 ±
2.9	*sll1483*		Periplasmic protein	5.5 ±
1.0	*ssl1633*	*hliC*	High light-inducible protein	5.5 ±
1.3	*sll0517*	*rbpA*	RNA-binding protein	5.4 ±
1.1	*sll0384*	*cbiQ*	ABC-type cobalt transport system permease protein	4.9 ±
0.0	*slr0400*		Putative inorganic polyphosphate/ATP-NAD kinase 1	4.7 ±
0.9	*sll2012*	*sigD*	RNA polymerase sigma factor	4.4 ±
0.8	*slr0955*		Putative tRNA/rRNA methyltransferase	4.4 ±
0.1	*slr0401*		Spermidine/putrescine transport system protein	4.4 ±
0.5	*slr1974*		Putative GTP-binding protein	4.4 ±
0.6	*slr1254*	*crtP*	Phytoene desaturase	4.3 ±
0.8	*slr1392*	*feoB*	Ferrous iron transport protein B	4.3 ±
1.2	*slr0399*	*ycf39*	Quinone-binding protein in PS II	4.3 ±
1.1	*slr0423*	*rlpA*	Rare lipoprotein A	4.1 ±
0.0	*sll1441*	*desB*	Fatty acid desaturase	4.0 ±
0.7	*sll0385*	*cbiO*	ABC-type cobalt transport system protein	4.0 ±
0.3	*slr1992*	*gpx2*	Glutathione peroxidase	3.7 ±
0.1	*slr0426*	*folE*	GTP cyclohydrolase I	3.7 ±
0.5	*sll1770*		ABC1-like	3.6 ±
1.3	*sll1147*		Glutathione S-transferase	3.6 ±
0.7	*sll1772*	*mutS*	DNA mismatch repair protein	3.6 ±
0.4	*slr1881*	*natE*	ABC-type neutral amino acid transport system	3.6 ±
± 5.7	*slr1544*		Hypothetical protein	20.6 :
± 4.0	*slr0082*		Hypothetical protein	15.4 :
0.9	*slr1927*		Hypothetical protein	6.6 ±
0.6	*ssr2016*		Hypothetical protein	6.2 ±
0.8	*sll1611*		Hypothetical protein	5.2 ±
0.5	*sll1911*		Hypothetical protein	5.1 ±
0.0	*slr1747*		Hypothetical protein	5.0 ±
0.1	*sll0494*		Hypothetical protein	4.5 ±
0.5	*sll0815*		Hypothetical protein	4.4 ±
0.5	*slr0236*		Hypothetical protein	4.4 ±
1.3	*slr1677*		Hypothetical protein	4.1 ±
0.6	*slr1436*		Hypothetical protein	4.0 ±
0.7	*sll0355*		Hypothetical protein	3.9 ±
1.7	*slr0616*		Hypothetical protein	3.8 ±
0.6	*sll0185*		Hypothetical protein	3.7 ±

ın 3.5 are listed. Cells that had been grown at 34°C were incubated at 22°C for 30 min. Genes with ratios of transcript levels greater tha

Table.1. Cold inducible genes in *Synechocystis* sp. PCC 6803 (Adapted from Inaba et al., 2003)

1.5. RNA helicases

1.5.1. Introduction on RNA helicases

RNA helicases are the enzymes that are active in modulating the secondary structure of RNAs by unwinding them in an ATP-dependent manner (Tanner and Linder, 2001). RNA helicases are ubiquitously distributed in all the biological kingdoms (Rocak and Linder, 2004). RNA helicases are responsible for modifying the secondary structures of mRNA, which is a critical factor in the regulation of translation (Fuller-Pace, 1994). RNA helicases utilize the free energy change of binding and hydrolyzing a nucleotide triphosphate to dissociate duplexes or displace bound proteins (Fig.1.6).

Reports have indicated that RNA helicase expression or activity is regulated not only with respect to participation in housekeeping processes but also in response to changes in specific environmental variables, including temperature, light, oxygen and osmolarity but also demonstrated that RNA helicases participate in various cellular processes involving RNA maturation functioning in ribosome biogenesis, RNA splicing, transport, and turnover, transcription, translation initiation, RNAi, RNA editing, and development (Rocak and Linder, 2004; Rossler et al., 2001). The involvement of RNA helicases in several cellular functions is indicated in the Figure1.7.

1.5.2. Biochemical activity of RNA helicases

Helicases are the DEAD- box proteins that are known to rearrange RNA duplexes and exhibit a directional activity; that is, either a 5’ to 3’ or 3’ to 5’ in an ATP-dependent manner. This activity can be envisaged in several ways and three non-exclusive models were presented below (Fig.1.8).

In a first model, the DEAD-box protein functions simply by an ‘on–off ’mechanism, which implies ‘breathing’ (that is, thermal denaturation of the duplex ends is used by the

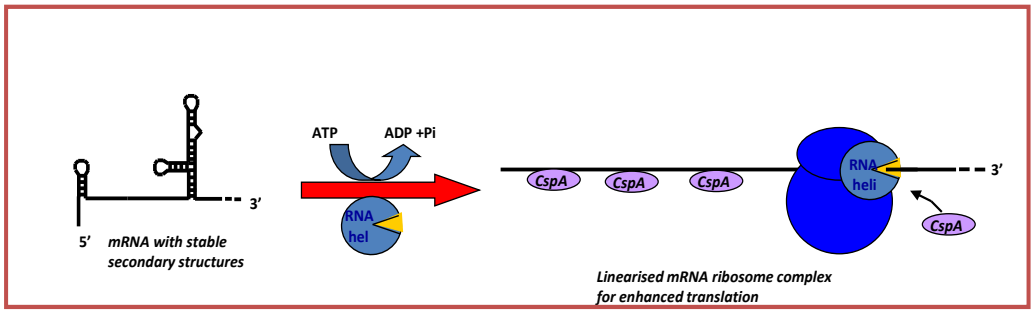


Fig.1.6. Model showing RNA helicases resuming the secondary structured mRNA and resume the protein synthesis under cold stress.

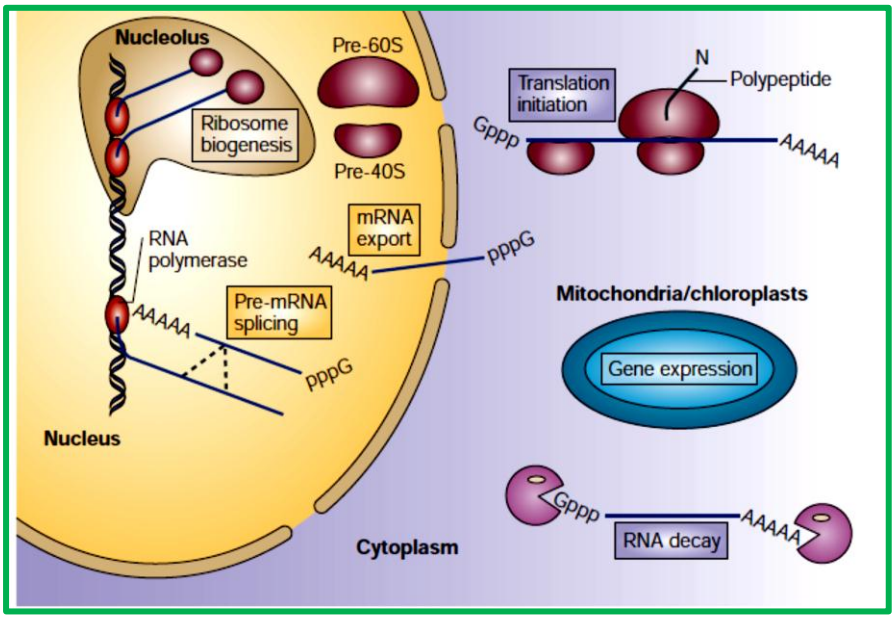


Fig.1.7. Cellular processes which require RNA helicases. Genetic and biochemical experiments in *Saccharomyces cerevisiae*, as well as in other organisms, have indicated several cellular functions for the RNA helicases. These include ribosome biogenesis *Fal1* (also known as eIF4AIII), *Rok1*, *Spb4* and *Dbp10*), pre-mRNA splicing, *Prp5* and *Sub2*, mRNA export *Dbp5* and *Sub2*, translation initiation *eIF4A*, *Ded1* and *Vasa*), organellar gene expression (*Mss116, Mrh4* and *Cyt-19*) and RNA decay *RhlB, Dbp2* and *Dhh1* (also known as *Xp54*)). It is thought that some DEAD-box proteins might function in two or more processes. In doing so, they probably serve as links or control elements for those cellular processes. Adapted from Rocak and Linder, 2004.

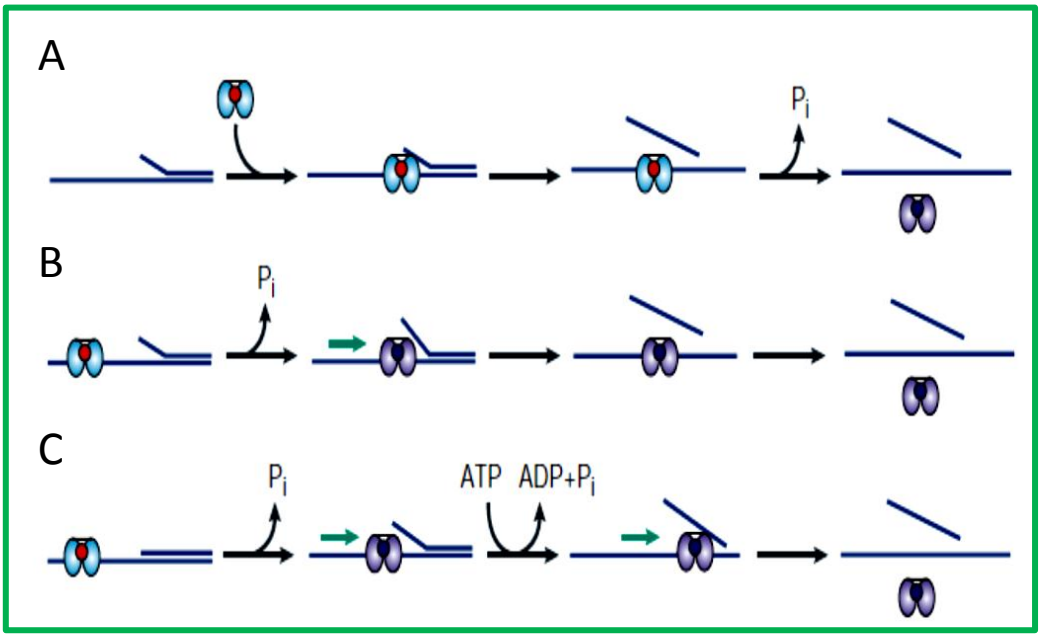


Fig.1.8. Possible modes of unwinding by DEAD-box RNA helicases. ATP-dependent unwinding can be envisaged in several ways. Here three distinct modes of unwinding, although there might be intermediate mechanisms. A). The first model (On and off) depicts a DEAD-box RNA helicase that associates and dissociates from a single-stranded RNA tail and might thereby destabilize a duplex. ATP hydrolysis is required to release the RNA. B). In the second model, (Translocation) the helicase is walking actively along the single-stranded RNA, and once it encounters the duplex, again, it destabilizes a breathing molecule. C). In the third model (Translocation and unwinding), the helicase uses energy from ATP hydrolysis for translocation and active unwinding. For simplicity, the enzyme is represented as a monomer with an ATP (red) or ADP (blue) in the cleft formed by domains 1 and 2. Pi, inorganic phosphate. Adapted from Rocak and Linder, 2004.

protein to attach to the open end). Such thermal breathing of a duplex has been estimated to occur at a rate of about 1,000 times per second. Once the protein is bound, the duplex is in an unstable state, which can result either in complete thermal melting or in dissociation of the protein from the RNA without unwinding. In this 'on–off' type of model, ATP hydrolysis is used to release the substrate from the protein to enable recycling, which is necessary for a new round of binding (Singleton et al., 2002; Chen et al., 1982).

In second model, DEAD-box proteins use the energy that is gained from ATP hydrolysis to translocate along single-stranded RNA. Once the protein encounters the duplex, it takes advantage of thermal melting to proceed along the transiently denatured strand to prevent re-annealing. In this model of passive unwinding, ATP hydrolysis is required for translocation to enhance the chance of denaturation by reducing the rate of re-annealing. Further thermal melting and translocation will finally result in dissociation of the duplex. Depending on the affinity of the helicase for the substrate, the activity can be more or less processive (Rozen et al., 1990).

In a third model, active destabilization of an RNA duplex requires the energy from ATP to power both translocation and active disruption of the base pairs at the junction of single-stranded and double stranded RNA(Singleton et al., 2002).

1.5.3. Motifs of RNA helicases

Alignment of helicase proteins that are required for a variety of processes in different organisms revealed eight conserved motifs, as well as a few conserved, but isolated, residues. One of the motifs (Asp-Glu-Ala-Asp, or DEAD in one-letter code) gave the protein family its name. Recently, an amino-terminal motif has been identified and named the Q-motif, which refers to a highly conserved glutamine residue in this motif (Tanner et al., 2003; Tanner, 2003). All motifs are conserved at similar positions in the members of the protein family, defining a highly conserved core element that is flanked by divergent amino- and carboxy-

terminal sequences. At present, over 500 different DEAD-box-protein sequences are present in protein databases (such as SwissProt and TrEMBL,) and despite these large numbers of proteins of various origins, the consensus sequences have remained essentially unchanged (Fig.1.9).

Considering these conserved motifs, DEAD-box proteins can be clearly distinguished from RNA helicases of related families, such as the DEAH-box or the SKI2 families. The alignments of members of these other families also show highly conserved motifs. Some of them are directly comparable to the motifs in the DEAD-box family, but others are not. Motif I, also known as the WALKER A motif, and motif II, which corresponds to the WALKER B motif, are present in all the families, and are recognizable as such (Walker et al., 1982). However, the striking difference in the DEAH box and Ski2 families is the presence of a histidine residue in place of the second aspartic acid in motif II.

Therefore, the RNA helicase families are often referred to as DExD/H proteins. Other motifs, such as motifs Ia or Ib, are more divergent. Significantly, so far, no helicase has been described that contains a mixture of motifs from different families. For example, the DEAD motif is always found together with the HRxGRxGR motif (motif VI), whereas DEAH is always associated with QRxGRxGR in the DEAH-box family. The newly discovered Q-motif and a conserved upstream aromatic residue are also highly characteristic of DEAD-box proteins. This motif was proposed to function as a sensor to determine the state of the bound ATP. It is interesting that the use of NTPs is different between DEAD-box and DEAH-box families: whereas DEAD-box proteins require ATP, DEAH-box proteins are more promiscuous in their use of NTPs.

Moreover, the respective motifs, as defined by the alignment of the members of each family, probably share their functional roles. It is intriguing that the families are so clearly

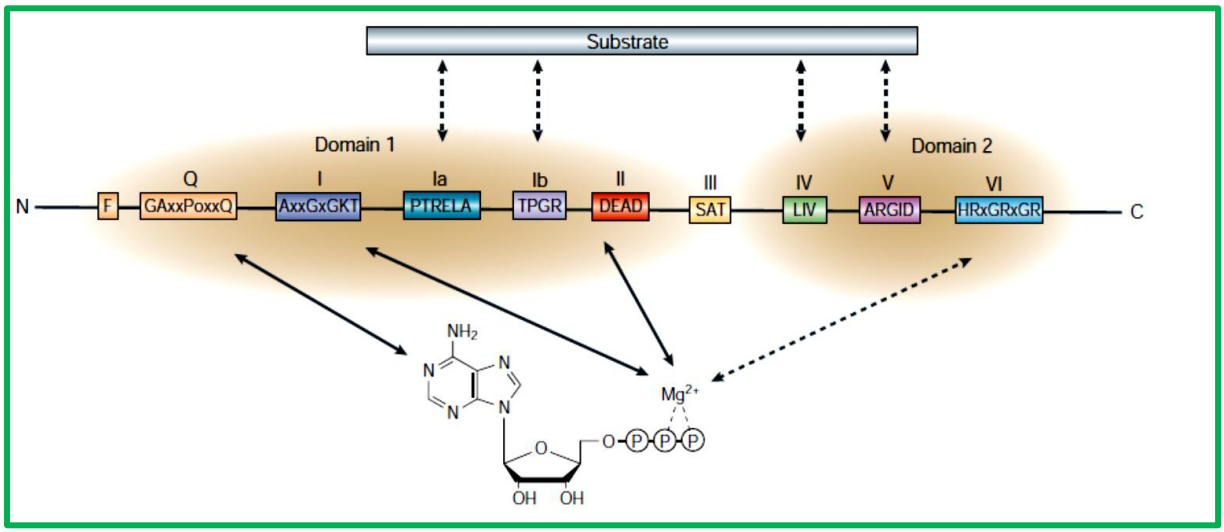


Fig.1.9. The conserved motifs of DEAD- box proteins and their interaction with ATP. A sequence alignment of the DEAD-box proteins has revealed nine conserved motifs. On the basis of mutational analyses and structural data, roles for many of the consensus elements have been postulated. The Q-motif and motifs I and II (WALKER motif A and B, respectively) bind ATP and are required for its hydrolysis. The highly conserved glutamine in the Q-motif forms hydrogen bonds with nitrogen atoms at positions 6 and 7 of the adenine, whereas the adjacent aromatic residue stacks with the adenine base. Both glutamine and serine/threonine (depicted as 'o' in the diagram) from the Q-motif form hydrogen bonds with conserved residues of motif I. It was proposed that the Q-motif, as well as the upstream phenylalanine residue, regulate ATP binding and hydrolysis. Motif I (AxxGxGKT) forms a loop structure (P loop) that accommodates the α and β phosphates of ATP; the lysine residue interacts with phosphates of MgATP or MgADP, whereas the threonine residue interacts with Mg^{2+}. Motif II (or the DEAD motif) forms interactions with the β and δ phosphates through Mg^{2+} and is required for ATP hydrolysis. In the eIF4A structure, motif II forms hydrogen bonds with the conserved residues of motif III in domain 1. Motif III links the ATP binding and hydrolysis to conformational changes that are required for helicase activity. Accordingly, mutations in motif III can separate NTP hydrolysis from the unwinding activity of DEAD-box and DEAH-box proteins. Motif VI is believed to participate in ATP binding, and mutations therein affect ATP hydrolysis. The structures of RNA helicases indicate that the remaining motifs (Ia, Ib, IV and V) are probably involved in RNA binding, although biochemical data are still lacking. Adapted from Rocak and Linder, 2004.

distinct, as this indicates that there must be some fundamental difference between these families.

1.5.4. Functional Diversity of Stress regulated RNA Helicases

The known RNA helicase genes whose expression is regulated in response to abiotic stress and their characteristics are summarized in Table 2.

1.5.4.1. Ribosome biogenesis

Physiological functions for stress-regulated RNA helicases have been identified in few cases. In *E.coli*, *SrmB* is required early in 50S ribosomal subunit assembly and although not reported to be a cold inducible protein, it has been proposed to rearrange an RNA secondary structure that is thermodynamically stabilized at low temperature (Charollias et al., 2003). It is reported that the cold-regulated helicase, CsdA, also performs a role in 50S subunit biogenesis in *E.coli*, although at a later stage than SrmB (Charollais et al., 2004).

CsdA performs multiple roles in translation initiation and ribosome maturation in response to cold stress, presumably involving rearrangement of RNA secondary structure (Jones et al., 1996). The proposed model for the involvement of CsdA for enhancement of translation is indicated in the Fig.1.10. In support of this proposal, CsdA has been shown recently to unwind dsRNA (Bizebard et al., 2004).

1.5.4.2. RNA turnover

CsdA also has the potential to function in RNA turnover through its ability to interact with the degradosome, a multi-subunit complex required for RNA turnover in organelles and prokaryotes. At 37^{o}C, the degradosome is composed of enolase, the RNases RNase E and PNPase, and the RNA helicase, RhlB which unwinds RNA secondary structure 3'to 5'in conjunction with PNPase-catalyzed RNA degradation (Purusharth et al., 2005). At 15^{o}C, CsdA can structurally and functionally replace RhlB in the degradosome complex *in vitro*, implying that cold stress results in the formation of a cold-adapted degradosome (Kressler et

Complex	Gene designation	Major proteins	Cofactors	Function
Photosystem II	*psb*	D1, D2, CP43, CP47, PsbO	Mn, Ca, Cl, Fe, PQ, chlorophyll, cyt b_{559}, pheophytin	Light-induced water splitting and PQ reduction
Succinate dehydrogenase	*sdh*	SdhA, SdhB, SdhC	Flavin, FeS centres	Succinate oxidation and PQ reduction
Type-1 NADPH dehydrogenase	*ndh*	NdhA-L	FeS centres	NADPH oxidation and PQ reduction
Cytochrome b_6f	*pet*	cyt b_6, cyt *f*, Rieske, subunit IV	2 cyt *b*, cyt *f* (cyt *c*), FeS	PQH_2 oxidation and PC/cyt c_{553} reduction
Photosystem I	*psa*	PsaA, PsaB and other Psa proteins	Chlorophyll, vitamin K_1, FeS centres	Light-induced PC/cyt c_{553} oxidation and Fd reduction
Cytochrome oxidase	*cta*	CtaC, CtaD, CtaE	Cu_A, Cu_B, Mg, cyt *a*, cyt a_3	Cyt *c* oxidation and O_2 reduction

Table 3. Major complexes involved with photosynthetic and respiratory electron flow in thylakoid membranes in cyanobacteria.

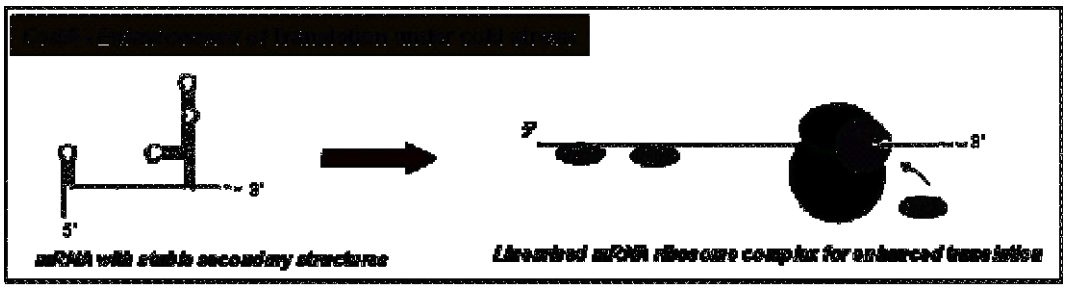


Fig.1.10. Model proposing the function of RNA helicase CsdA in rearranging the stabilized secondary RNA for enhanced translation. Stabilized, non-functional RNAs are recognized and unwound by the abiotic stress (cold stress in this example)-induced RNA helicase. Cold-induced Csps, potentially bind to the RNA helicase-generated ssRNA, thereby inhibiting spontaneous reversion to dsRNA, and permit translation initiation to proceed.

al., 1999). CsdA may be required to promote degradation of RNA whose secondary structure is stabilized by low temperature to a degree where RhlB is unable to unwind the structure. Although this is an interesting proposal, the *in vivo* physiological relevance of this interaction requires further investigation (Fig.1.11).

Binding studies indicate that RNase E has the potential to associate with SrmB, RhlE and CsdA *in vitro,* with RhlB, RhlE and CsdA being interchangeable with respect to the facilitation of structured RNA degradation (Kressler et al., 1999; Khemici et al., 2004). In support of the cold-adapted degradosome proposal, biochemical purification has also indicated that RhlE is a component of the degradosome in the cold-adapted, Antarctic bacterium, *Pseudomonas syringae* Lz4W (Purusharth et al., 2005). These results indicate the potential for degradosome cold adaptation by association with a specific cold-induced RNA helicase in a range of bacteria, highlighting the requirement for RNA helicase activity to maintain RNA turnover during cold stress.

1.5.4.3. Cell cycle progression

Evidence that the yeast RNA helicase DED1 also performs roles in addition to its function in translation initiation was obtained by the observation that *ded*1 inactivation leads to cell cycle arrest (Liu et al., 2002). DED1 physically interacts with the protein kinases, Chk1 and Cdc2, required for cell cycle response to DNA damage and the primary cyclin-dependent kinase regulating cell cycle progression, respectively (Liu et al., 2002). Unfortunately, the physiological function of the DED1–Chk1/Cdc2 interaction *in vivo* is unknown.

1.5.4.4. Transcriptional regulation

There is one instance which links RNA helicase activity with the regulation of transcription. c-Jun is a transcriptional regulator activated by phosphorylation in response to a range of external stimuli including abiotic stress and differentiation signals. It has been identified that

the human DEAD-box RNA helicase RHII/Gu interacts with c-Jun independently of c-Jun-mediated phosphorylation (Westermarck et al., 2002).

1.5.4.5. Nuclear mRNA export

mRNA transport through the nuclear pore complex is a frequent target of stress response mechanisms. The yeast DEAD-box RNA helicase, Dbp5p/Rat8p, is required for mRNA export from the nucleus (Snay-Hodge et al., 1998). Dbp5p/ Rat8p shuttles between the nucleus and cytoplasm and is required to dissociate the nuclear mRNA export receptor, Mex67p, during transfer through the nuclear pore complex (Lund and Guthrie, 2005). Interestingly, genetic analysis of the cold-sensitive defect observed in *rat*8-7 mutants suggests that the requirement for the Mex67p-clearing activity of Dbp5p/Rat8p is enhanced at low temperature (Lund and Guthrie, 2005).

Recently, a temperature-regulated RNA helicase, LOS4, has been linked with developmental processes including flowering and vernalization in *Arabidopsis* (Gong et al., 2002). Similar to bacteria and yeast, temperate plant cold acclimation involves induction of specific cold-responsive (COR) genes. COR gene expression is activated by the C-repeat-binding factor (CBF) family of transcriptional activators which are also rapidly and transiently induced at low temperature (Fig.1.12). The allelic *Arabidopsis* RNA helicase mutants, *los*4-1 and *los*4-2/ cryophyte, reveal that the LOS4 RNA helicase is an early regulator of CBF transcription factor expression in response to plant chilling (Gong et al., 2002). LOS4 inactivation renders the plants sensitive to chilling although the two mutants differentially affect the CBF–COR cold response pathway; *los*4-1 reduces whereas *los*4-2 enhances the expression of CBFs and their downstream target genes. This divergent response is mediated through a differential effect on nuclear mRNA export that *los*4-1 inhibits and *los*4-2 enhances at low temperature (Gong et al., 2002). The conventional explanation is that the lack of CBF expression in los4 mutants derives from a direct effect on CBF mRNA

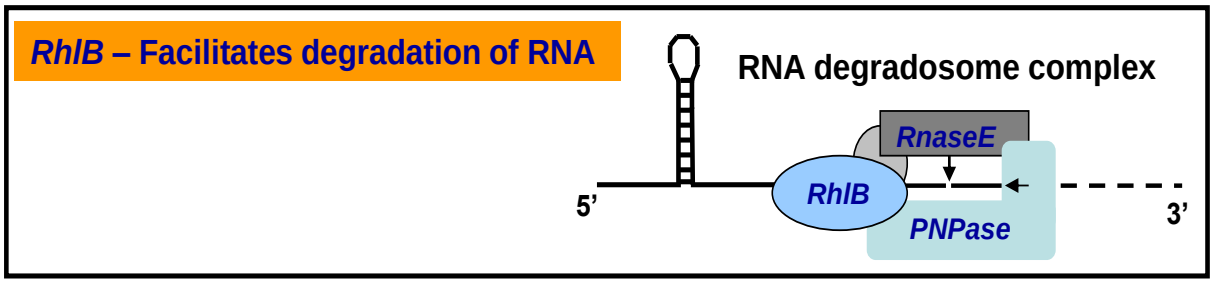


Fig.1.11. Model showing the degradosome complex comprising of enolase, RNases, RNase E and PNPase, RNA helicase, RhlB is an RNA helicase which unwinds RNA secondary structure from 3'to 5'in conjunction with PNPase-catalyzed RNA degradation.

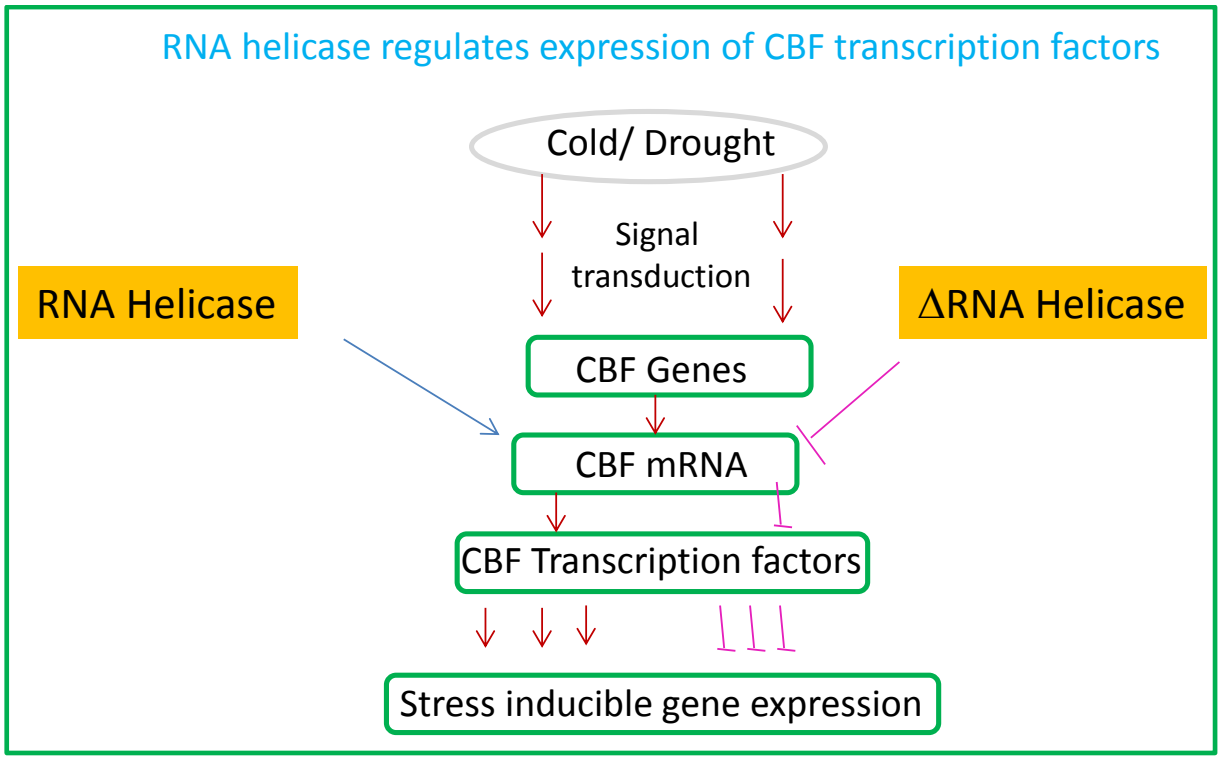


Fig.1.12. Model representing the function of LOS4 RNA helicase as an early regulator of CBF transcription factor expression in response to plant chilling. LOS4 inactivation renders the plants sensitive to chilling effecting CBF–COR cold response pathway inhibiting the nuclear mRNA export at low temperatures.

export and the corresponding lack of CBF protein. However, a variety of observations indicate that LOS4 may perform more specific roles in low temperature response, unrelated to its function in mRNA export.

1.5.4.6. RNA helicases in m-RNA stability under low temperatures

A significant aspect affecting RNA helicase expression in response to cold stress is the regulation of transcript stability. *crhC* transcripts in *Anabaena* are significantly stabilized in response to a temperature downshift (Chamot and Owttrim, 2000). Although the mechanism remains unknown, mRNA stabilization is an important factor regulating expression of other low temperature-induced genes including *cspA*, *pnp*, *hupB* and *infB* (Gualerzi et al., 2003). Stabilization of the *E.coli cspA* gene requires the 5'-untranslated region (5'-UTR), which is unusually long with respect to the average bacterial mRNA (Fang et al., 1997). It is presumed that destabilization arises through the action of a nuclease, potentially RNaseE, followed by PNPase degradation (Fang et al., 1997).

Thus, low temperature-induced RNA helicase expression involves a combination of both *cis*- and *trans*-acting factors, with mRNA stabilization performing a crucial role in transcript accumulation. Enhancement of mRNA stability would restrict helicase activity to conditions where the cell experiences the specific abiotic stress, providing the ability to coordinate target RNA function downstream of helicase synthesis.

1.5.4.7. RNA helicases in translation initiation

Genome-wide transcript profiling in *Saccharomyces cerevisiae* identified the RNA helicases *DED*1 and *Dbp*2 as early cold response genes (Schade et al., 2004). *DED*1 and *Dbp*2 are involved in translation initiation (Chuang et al., 1997; Cruz et al., 1997) and nonsense-mediated mRNA decay and rRNA processing (Bond et al., 2001), respectively, thereby linking helicase activity with the maintenance of these processes during low temperature stress.

1.5.4.8. Other functions

In plants, two DEAD-box-related helicases, termed pea DNA helicase 47 (PDH47) and PDH45 are induced by a variety of abiotic stresses, suggesting that they are components of a general stress response mechanism. PDH47 expression is differentially induced in a tissue specific manner with induction by cold and salinity stress in shoots and roots and heat and ABA treatment in roots (Vashist et al., 2005). PDH47 was localized to both the cytosol and nucleus, suggesting shuttling between the compartments (Vashist et al., 2005). The second characterized helicase, PDH45 is a unique member of the DEAD-box family of RNA helicases as it contains DESD and SRT instead of the characteristic DEAD and SAT motifs (Pham et al., 2000). PDH45 transcript is induced in pea seedlings in response to a range of abiotic stresses including salt (specifically Na+), dehydration, wounding and low temperature, leading to the suggestion that pdh45 transcript accumulates in response to general water stress caused by desiccation(Sanan-Mishra et al., 2005). The physiological importance and conservation of PDH45 function in the salt-stress response was demonstrated by the observation that constitutive expression of PDH45 conveys salt tolerance in tobacco (Sanan-Mishra et al., 2005). These results imply that PDH45 performs a crucial function, directly involved in cellular response to a specific abiotic stress.

Higher plant genomes encode a number of RNA helicase genes (Aubourg et al., 1999) whose expression and polyadenylation patterns are tissue specific (Owttrim et al., 1991; Owttrim et al., 1994; Brander et al., 1995).

1.5.4.9. Cold regulated expression of RNA helicases

Temperature fluctuation is most frequently encountered by living organisms and temperatures below those required for optimal growth elicit the cold shock response, leading to the de novo synthesis of cold-induced proteins (CIPs). The RNA helicase genes, *crhC*, *csdA* and *deaD*, whose expression is regulated by a reduction in growth temperature, have been characterized

in the photosynthetic cyanobacterium *Anabaena* sp. strain PCC 7120 (Chamot et al., 1999; Chamot and Owttrim, 2000), *Escherichia coli* (Jones et al., 1996), and the cold-adapted Antarctic methanogenic archaeon, *Methanococcoides burtonii* (Lim et al., 2000), respectively. Mechanisms involved in low temperature regulated expression have been investigated for *crhC* in *Anabaena* whose expression is low temperature specific as a range of additional abiotic stresses do not induce transcript accumulation (Chamot et al., 1999). Induction is not transient and the mechanism involves low temperature enhancement of *crhC* transcript stability (Chamot and Owttrim, 2000), a common theme in cold shock gene expression (Gualerzi et al., 2003). CrhC was also found to be is tightly associated with the plasma membrane, pre-dominantly at the cell poles (El-Fahmawi and Owttrim, 2003), implying a role in RNA metabolism that preferentially occurs at this location during cold stress. Expression of cold-regulated RNA helicase genes have also been identified by transcriptional profiling in the hyperthermophilic methano archaeon, *Methanococcus jannaschii* (Boonyaratanakornkit et al., 2005) and the gram-positive bacterium, *B.subtilis* (Beckering et al., 2002). Genetic analysis of the *Bacillus* genes, *cshA* and *cshB*, indicated inactivation of both is lethal, emphasizing the requirement for RNA helicase activity during cold stress (Hunger et al., 2006).

Furthermore, it has been revealed that CshB colocalizes with the RNA binding cold shock protein (CSP), CspB, during active transcription. These observations position cold-induced RBPs and RNA helicases in close proximity with mRNA, implying an active role in translation initiation at low temperature. Related to the polar localization of CrhC, a portion of the cellular CshB localizes to the cell poles in *Bacillus*, although it is also distributed throughout the cell (Hugner et al., 2006). It is interesting to note that all of the cold-regulated RNA helicase genes discussed above encode proteins belonging to the DEAD-box family.

All the above studies are consistent with the hypothesis that the RNA secondary structure rearrangement activity provided by a cold-regulated helicase is an ancient and universal requirement for normal cellular function at reduced temperature.

1.6. Model organism *Synechocystis*

The unicellular cyanobacteria have several features that make them particularly suitable for studies on stress responses at the molecular level. The general features of the plasma and thylakoid membranes of cyanobacterial cells are similar to those of the chloroplasts of higher plants in terms of lipid composition and the assembly of membranes. Therefore, cyanobacteria can be expected to serve as powerful model systems for studying the molecular mechanisms of the responses and acclimation to stress (Murata and Wada, 1995; Los and Murata, 2004), also these mechanisms may provide models that are applicable to plants as well. Some strains of cyanobacteria, such as *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7942, and *Synechococcus* sp. PCC 7002, are naturally competent and thus, foreign DNA is incorporated into cells and is integrated into their genomes by homologous recombination at high frequency (Williams, 1988; Haselkorn, 1991). As a result, cyanobacteria are widely used by researchers for the production of mutants with disrupted genes of interest (Vermaas, 1998).

The entire nucleotide sequence of the genome of *Synechocystis* was determined by Kaneko et al., (1996) as the first sequence of a cyanobacterial genome to be reported. Subsequently, the entire sequences of all four of the plasmids harboured by *Synechocystis* were reported by this group (Kaneko et al., 2003). Genome sequences provide vast amounts of basic information, which can be exploited for genome-wide studies of gene expression. In 1999, Takara Bio Co. (Ohtu, Japan) initiated the production of a genome-wide cDNA microarray for the analysis of gene expression in *Synechocystis*. Their DNA microarray covers 3,079 (97%) of the 3,165 genes on the chromosome of *Synechocystis* (99 genes for

transposases are excluded from this calculation) but the microarray does not include genes from the four plasmids. The original results of analysis of patterns of gene expression in this cyanobacterium can be found in the KEGG expression database (List of experimental data available at http://www. genome.jp/kegg/expression/).

1.6.1. Redox regulated RNA helicase CrhR

Since fluctuation in light quality and quantity is a stress frequently encountered by photosynthetic organisms, ability to sense and respond to dynamic light conditions is crucial for their survival. The cyanobacterium *Synechocystis* has an RNA helicase CrhR Expression of *crhR*, from the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803, is regulated by light-driven changes in the redox status of the electron transport chain (Kujat and Owttrim, 2000). CrhR has also been characterized as being induced by cold and salt stress (Chamot et al., 2005; Vinnemeier and Hagemann, 1999). In cells grown under constant illumination, there is an enhanced reduction of the photosynthetic electron transport chain, observed in response to these stress factors, presumably contributes to the observed increase in *crhR* transcript accumulation (Savitch et al., 2001). This regulation implies that CrhR functions in an aspect of RNA metabolism related to survival in the light, presumably involving maintenance of optimal photosynthetic capacity, either light harvesting or carbon acquisition and fixation.

The main focus of the present study is on the RNA Helicase *crhR* in *Synechocystis* which is specifically induced upon low temperature treatment. The aim of this thesis is to generate a cyanobacterial DEAD box RNA helicase *crhR* mutant in the cyanobacterium *Synechocystis* sp.PCC 6803 and to elucidate its functional role in photosynthetic acclimation under low temperature stress.

1.7. Objectives

1. Mutagenesis of the gene coding for RNA helicase, *crhR* in *Synechocystis* sp. PCC 6803 and phenotype characterization of the mutant.

2. Effect of *crhR* mutation on low temperature induced changes in photosynthetic pigment protein complexes.

3. Effect of *crhR* mutation on low temperature induced functional balancing between photosystems.

4. Role of RNA helicase in regulation of low temperature inducible gene expression for optimized photosynthesis.

1.8. Cyanobacteria photosynthesis and respiration

1.8.1. Cyanobacteria are different from that of higher plants

The combination of photosynthesis and respiration in a single compartment in cyanobacterial cell is thought to be quite unique. Photosynthesis and respiration in cyanobacteria require electron transport pathways that to a large extent are catalysed by protein complexes in membranes. Figure1.13 illustrates the compartmentalization of the cyanobacterial cell. The thylakoid membrane, the internal membrane system that separates the cytoplasm from the lumen and that is present in virtually all cyanobacteria, contains both photosynthetic and respiratory electron transport chains. These electron transport chains intersect, and in part utilize the same components in the membrane. It should be noted oxygenic photosynthesis (conversion of CO_2 and water to sugars using the energy from light) essentially is the reverse of respiration (conversion of sugars to CO_2 and water releasing energy). The cytoplasmic membrane, separating the cytoplasm from the periplasm, contains a respiratory electron transport chain but not photosynthetic complexes in most cyanobacteria. Therefore, in most cyanobacteria, photosynthetic electron transport occurs solely in thylakoids, whereas

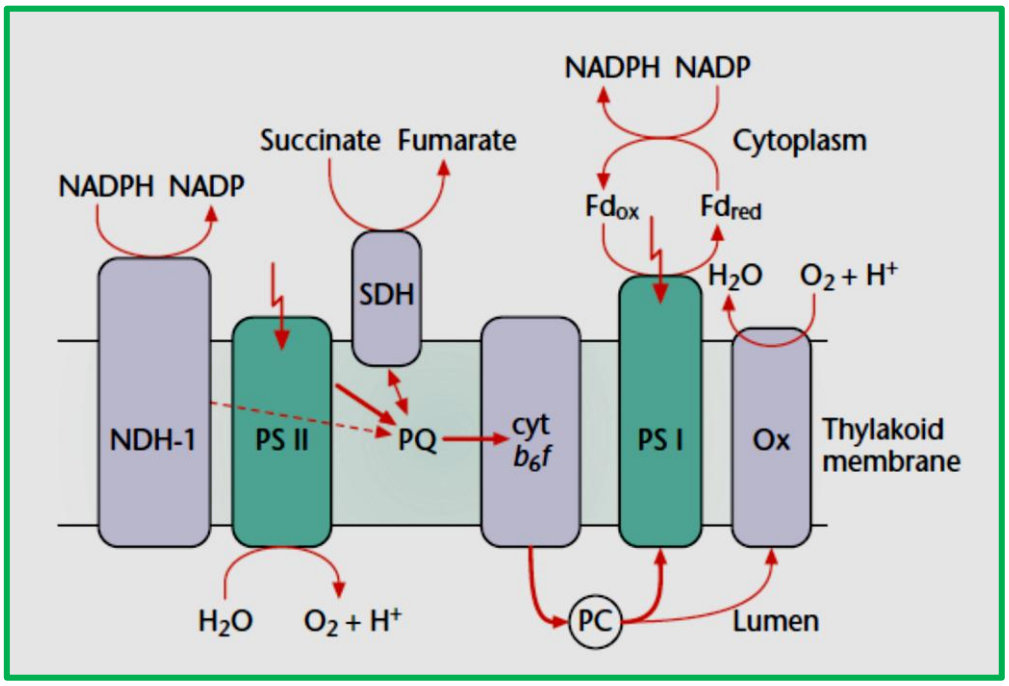


Fig.1.13. Schematic representation of the intersecting photosynthetic and respiratory electron transport pathways in thylakoid membranes of the cyanobacterium *Synechocystis* sp. PCC 6803. Arrows indicate electron transfer reactions, and thunderbolts designate light that sets into motion the redox reactions in the two photosystems. The thickness of each arrow is an approximate indication of the rate of the corresponding reaction. Electron transfer complexes that are specifically involved in photosynthetic electron transfer are PSII and PSI, whereas those specific for respiratory electron flow include NDH-1, SDH, and the terminal oxidase. PQ, Cyt b_6f and PC are shared by both pathways. The electron transfer arrows involving SDH are drawn in both directions, as the difference in midpoint redox potentials between the PQ/PQH_2 and fumarate/succinate redox couples is small and therefore electron flow can occur in both directions depending on the relative concentrations of PQ, PQH_2, succinate and fumarate. Abbreviations: cyt b_6f, the cytochrome b_6f complex; Fd ox and Fd red,ferredoxin in oxidized and reduced forms, respectively; NADP(H), nicotinamide – adenine dinucleotide phosphate (reduced form); NDH-1, type 1 NADPH dehydrogenase; Ox, terminal oxidase; PC, plastocyanin; PQ, plastoquinone; photosystem I, PSI; photosystem II, PSII; SDH, succinate dehydrogenase. Adapted from Vermaas et al ., 2001.

respiratory electron flow takes place in both the thylakoid and cytoplasmic membrane systems.

The presence and simultaneous activity of photosynthetic and respiratory electron transport chains in the same membrane system is unusual. A schematic representation of the respiratory and photosynthetic electron transport chains in cyanobacterial thylakoid membranes is shown in Figure 1.13. As indicated, cyanobacteria utilize several redox active components in thylakoids for both photosynthesis and respiration, including the plastoquinone (PQ) pool, the cytochrome b_6f complex, and the soluble electron carriers in the lumen.

1.8.2. Photosynthetic electron flow

The photosynthetic electron transport chain in cyanobacteria is identical to that in plants, even though some of the polypeptides that are part of electron transport appear to be of different evolutionary origin in the two systems. Indeed, chloroplasts in plants are thought to have originated from cyanobacterial ancestors. The photosynthetic electron transport chain of oxygenic organisms has been reviewed extensively (Ort and Yocum, 1996; Hippler et al., 1998; Whitmarsh, 1998). As indicated in Figure 1.13, PSII uses light energy to split water and to reduce the PQ pool. Table.3 summarizes the protein components involved with PSII and with other complexes mentioned in Figure 1.13. Electrons are transported from the PQ pool to the cytochrome b_6f complex and from there to a soluble electron carrier on the luminal side of the thylakoid membrane. In cyanobacteria this soluble carrier may be plastocyanin or cytochrome c553, depending on the species and on the availability of copper (plastocyanin is a copper containing enzyme). Either of these soluble one-electron carriers can reduce the oxidized PSI reaction centre chlorophyll, P700. This oxidized form of the reaction centre chlorophyll is formed by a light-induced transfer of an electron from PSI to ferredoxin (Fd) and eventually to NADP. Reduced NADP can be used for CO_2 fixation.

Complex	Gene designation	Major proteins	Cofactors	Function
Photosystem II	*psb*	D1, D2, CP43, CP47, PsbO	Mn, Ca, Cl, Fe, PQ, chlorophyll, cyt b_{559}, pheophytin	Light-induced water splitting and PQ reduction
Succinate dehydrogenase	*sdh*	SdhA, SdhB, SdhC	Flavin, FeS centres	Succinate oxidation and PQ reduction
Type-1 NADPH dehydrogenase	*ndh*	NdhA-L	FeS centres	NADPH oxidation and PQ reduction
Cytochrome b_6f	*pet*	cyt b_6, cyt *f*, Rieske, subunit IV	2 cyt *b*, cyt *f* (cyt *c*), FeS	PQH_2 oxidation and PC/cyt c_{553} reduction
Photosystem I	*psa*	PsaA, PsaB and other Psa proteins	Chlorophyll, vitamin K_1, FeS centres	Light-induced PC/cyt c_{553} oxidation and Fd reduction
Cytochrome oxidase	*cta*	CtaC, CtaD, CtaE	Cu_A, Cu_B, Mg, cyt *a*, cyt a_3	Cyt *c* oxidation and O_2 reduction

Table 3. Major complexes involved with photosynthetic and respiratory electron flow in thylakoid membranes in cyanobacteria.

Photosynthetic electron transfer leads to a proton gradient across the thylakoid membrane. In PSII, protons are released into the lumen upon water splitting, and protons formed upon plastoquinol oxidation by the cytochrome b_6f complex are released into the lumen as well. The proton gradient across the thylakoid membrane is used for ATP synthesis by the ATP synthase in the thylakoid; this ATP may be applied for CO_2 fixation and for other cell processes.

One important difference between cyanobacteria and plants is that the stoichiometry of PSI and PSII in at least some species of cyanobacteria is much larger than 1, whereas in higher plants an equal amount of PSI and PSII is the rule. For example, in the cyanobacterium *Synechocystis* sp. PCC 6803 the PSI/PSII ratio is about 5 (Shen et al., 1993). One possible explanation for this unusual stoichiometry in cyanobacteria is the involvement of PSI in a significant amount of cyclic electron flow around this photosystem, in which electrons would flow from PSI/Fd back to PQ and cytochrome b_6f, and from there to PSI again (Bendall and Manasse, 1995). This electron transport pathway should contribute to a proton gradient, which could be used for ATP synthesis, but would not lead to net NADP reduction. However, a high rate of cyclic electron flow around PSI in cyanobacteria was not demonstrated experimentally. Another and probably more plausible reason for the relatively large amount of PSI in cyanobacteria is the abundance of respiratory electron transfer pathways into the PQ pool, whereas the capacity of respiratory electron flow out of the PQ pool may be more limited. Therefore, an abundance of PSI may guarantee a rather oxidized PQ pool in the light, which is important to minimize photodamage (Andersson and Barber, 1996). Moreover, the high amount of PSI may serve to compete effectively with cytochrome oxidase for electrons when light is available, thus maximizing the number of electrons that can be used for CO_2 fixation.

1.9. Cyanobacterial photosynthetic complexes and their strucuture

In cyanobacteria, higher plants and green algae, light energy is converted into the chemical energy by the action of different protein complexes, phycobilisomes (PBS), PS II, cytochrome b_6f, PSI and ATP synthase (Frasch, 1994) (Fig.1.14).

1.9.1. PBS

In cyanobacteria, there are specific light harvesting antenna complexes, PBS, which consist of phycobiliproteins (PBPs), (Fig.1.14). In the cyanobacterium *Synechocystis* there are two blue-pigmented PBPs: phycocyanin (PC) (Abs max 620 nm) and APC (Abs max 650 nm). Some cyanobacteria also have phycoerythrin (PE), a red-pigmented phycobiliprotein (Abs max 560 nm). The PBS contains core and rod substructures. PE and PC form the rods, which efficiently absorb and transfer excitation energy to the PBS core, which consist of predominantly APC (Bryant, 1994). The PBS core also contains terminal emitters (Abs max 683 nm), which tunnel light energy from the PBS to the RCs. The exact interaction between the PBS and the photosystems is still under debate. PBS excitation energy distribution between the two photosystems is regulated, in order to optimize photosynthetic performance (Mullineaux, 2008). PBS are also subject to carotene-triggered dissipation of absorbed energy as heat. The phenomenon is known as non-photochemical quenching (NPQ) in cyanobacteria (Rakhimberdieva et al., 2010).

The major PBS components are the chromophore-bearing biliproteins: PC, APC and PE. The last one is not present in the *Synechocystis* sp.6803 (Glazer, 1985). Minor components of PBS are linker polypeptides that are involved in the regulation of PBS quaternary structure and optimization of the energy transfer by modifying the light absorption properties of the PBPs (de Marsac and Cohen-Bazire, 1977; Mac Coll, 1998). The core-membrane linker L_{CM} with a molecular weight of about 99 kDa participates in the energy transfer from PBS to PSII. The rod-core linkers, L_{RC}, attach the peripheral rods to the core of

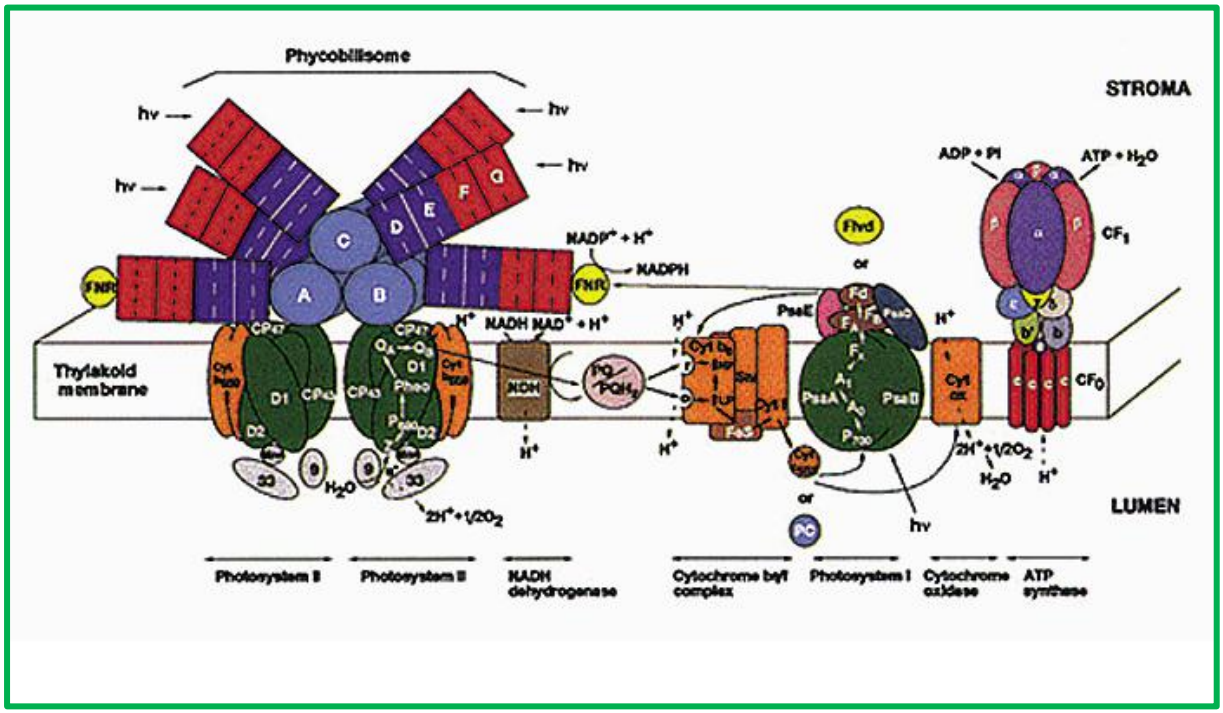


Fig.1.14. Schematic representation of the thylakoid membrane of cyanobacteria (adapted from Frasch, 1994).

PBS. In addition, rod linkers, L_R, and core linkers, L_C, are involved in the assembly of rods and core domains of PBS, respectively. Still, the question about the mechanisms of energy absorption and transfer to the photosystems in cyanobacteria has not been clarified. A major step of energy transfer in cyanobacteria includes transfer from PC of rods to APC in the cores and then from APC to the photosystem via the L_{CM} linker protein (Houmard et al., 1990; Macoll, 1998). Also, the mechanism that provides the energy distribution from PBS between PSI and PSII is still not clear. In favour is the model that PBS are mainly attached to dimer PSII particles and transfer energy directly PSII (Kuhl et al., 1999). However, recent studies in *Spirulina* cells showed that 20% of PBS are bound to PSII, while 60% of PBS transfer the energy to the PSI trimer and 20% are associated with PSI monomers (Rakhimberdieva et al., 2001).

1.9.2. PSII

PSII is a pigment-protein complex of the thylakoid membrane of oxygenic photosynthetic organisms. It catalyzes the light-induced electron transfer from water to PQ, with associated production of molecular oxygen. PSII is a large complex with polypeptide species, most of which are integral membrane proteins. A number of extrinsic proteins are also associated to PSII. The entire set of electron transfer cofactors, including chlorophyll *a*, pheophytin a, PQs, and non-heme iron, is associated with the D1/D2 heterodimer. These two proteins, together with the α- and ß-subunits of cytochrome b_{559} and the PsbI protein, constitute the so-called reaction centre II (RCII), which is the smallest PSII subparticle still able to perform light-induced charge separation. Two large integral membrane proteins, CP43 and CP47, each coordinating a number of chlorophyll *a* molecules, and several low molecular mass (<10 kDa) polypeptides are constituents of the PSII core complex, which is very similar in higher plants and cyanobacteria (Diner et al., 2002; Yu and Vermaas, 1993; Szabo et al., 2001; Kashino et al., 2002).

1.9.3. Cytochrome b_6f complex

The cytochrome b_6f complex provides the electronic connection between reaction centres of the PSI and of PSII and generally contributes to the transmembrane electrochemical proton gradient for adenosine triphosphate synthesis (Rich and Bendall, 1980). In cyanobacteria, the cytochrome b_6f complex is located both in thylakoid and cytoplasmic membranes. It is involved in both photosynthetic and respiratory electron transport, acting as a plastoquinol-cytochrome c_6-plastocyanin oxidoreductase and playing a role in electron transfer from PSII or NAD(P)H dehydrogenase to PSI or cytochrome oxidase, respectively. In all organisms capable of oxygenic photosynthesis the cytochrome b_6f complex consists of four major proteins and additional small subunits (Zhang et al., 2003). The 25 kDa cytochrome b_6 protein contains two b-type (Widger et al., 1984) and one novel x-type (Kurisu et al., 2003) hemes, and together with the subunit IV of 17 kDa, is homologous to cytochrome b of the cytochrome bc_1 complex (Widger et al., 1984). Cytochrome b_6 and subunit IV are integral membrane proteins with four and three predicted transmembrane -helices, respectively. Cytochrome f is a 31 kDa c-type cytochrome with a covalently bound heme in the large lumen-exposed domain; it is anchored by a single C-terminal -helix in the membrane. Similarly, the Rieske iron-sulfur protein has a large hydrophilic lumenal domain attached to a single transmembrane -helix at the N-terminus (Kallas, 1994). The genome of *Synechocystis* contains three open reading frames, designated *sll1316* (*petC1*), *slr1185* (*petC2*), and *sll1182* (*petC3*), encoding for three putative Rieske iron-sulfur proteins (Schneider et al., 2002).

1.9.4. PSI

PSI represents a multimeric polypeptide complex, formed basically around the PsaA/B heterodimer that binds 90 - 120 chlorophyll *a* molecules (Golbeck, 1994; Ben-Shem et al., 2003). The cyclic electron transport around PSI play a role in the generation of ATP required

for respiration of cyanobacteria and plants during dark stages (Howitt et al., 2001). Comparison of higher plant PSI with that of cyanobacteria showed that cyanobacteria do not contain the PsaG, PsaH, PsaN and PsaO proteins (Sener et al., 2005). On the other hand, PsaM protein has been identified only in cyanobacteria, but not in higher plants. PsaM is a low molecular weight protein with a single trans-membrane helix. The function of this protein is still not clear (Scheller et al., 2001; Sener et al., 2005). PSI can exist in two different forms: monomeric and trimeric. The trimeric form is a consequence of the association of monomeric PSI through the PsaL protein (Chitnis and Chitnis, 1993).

1.9.5. ATP synthase complex

The ATP synthase is a multisubunit membrane-bound protein complex, which catalyzes the synthesis of ATP from ADP and phosphate (Pedersen and Amzel, 1993; Frasch, 1994; Neisser et al., 1994; Dimroth et al., 2000). The activity of ATP synthase requires a proton gradient ΔpH and depends on the membrane potential Δψ (Kaim and Dimroth, 1999; Wieczorek et al., 1999; Dimroth et al., 2000). It is comprised of two subcomplexes, a membrane-integral part, F_0, and an extrinsic appendix, F_1. F_1F_0 ATP synthases comprise a huge family of enzymes with members found in the cytoplasmatic membrane of bacteria, inner membrane of mitochondria and thylakoid membranes. The hydrophilic F_1 component of the enzymes, which catalyzes the ATP synthesis, is comprised of five subunits (α, β, γ, δ, and ε). The hydrophobic F_0 component, made up of four subunits (a, b, b´ and c), forms the proton channel through the membrane (Frasch, 1994; Robertson et al., 1989). The gene coding for ATP synthase subunits are generally encoded by two operons. The atp1 operon encodes the subunits a, c, b´, b, δ, α, γ. The atp2 operon contains the genes for subunits β and ε. The *Synechocystis* F_1F_0 ATP synthase is also composed of nine subunits (Lill and Nelson, 1991). The cytoplasmic membrane of cyanobacteria contains some additional type of ATP synthase called P-type ATPase (P-ATPase), present in small amounts. It increases during

light-limiting growth when respiration becomes more important for energy generation (Niesser et al., 1994).

1.9.6. Aim of the study

Changes in global environment might lead to stress conditions in photosynthetic organisms and limit the efficiency of photosynthesis. Understanding photosynthesis and its protective mechanisms might lead to the development of plant, algae and cyanobacteria with improved growth characteristics even under stress conditions. Cyanobacteria, our model organism, are regarded as an origin of the plant chloroplast; therefore is an excellent organism to study higher plant photosynthesis. RNA Helicases are found to be very important in cellular function especially during abiotic stress conditions. However their importance in photosynthesis under low temperature conditions is not known. Their functional features involving the protective mechanisms are not completely elucidated yet. Complete genome sequences and the transformability of cyanobacterium *Synechocystis* allow us to generate various low temperature inducible mutants and study the importance of RNA helicase mutant in photosynthetic processes. Therefore, the aims of my thesis are:

1. Mutagenesis of the gene coding for RNA helicase, *crhR* in *Synechocystis* sp. PCC 6803, phenotype characterization of the mutant

2. Effect of *crhR* mutation on low temperature induced changes in photosynthetic pigment protein complexes.

3. Effect of *crhR* mutation on low temperature induced functional balancing between photosystems.

4. Role of RNA helicase in regulation of low temperature inducible gene expression for optimized photosynthesis.

I approached the research question by constructing inactivation strain of *crhR*. The importance of CrhR during low temperature acclimation was investigated by measuring the performance of the inactivation strains under low temperature conditions. In addition to physiological characteristics, altered phenotype under low temperature was studied.

Particular attention was paid to the investigation of the involvement of long term and short term of regulatory mechanisms involving energy redistribution under low temperature in *Synehocystis.*

Experimental procedures

Chapter 2

2. Materials and methods

2.1. Strains and growth conditions for *Synechocystis*

A strain of *Synechocystis* sp.PCC 6803 which is tolerant to glucose (Williams, 1988), was originally obtained from Dr. J. G. K. Williams (Dupont de Nemours). Wild-type cells were grown at 34^{o}C in BG-11 medium (Stanier et al., 1971), which had been buffered with 20 mM HEPES-NaOH (pH 7.5), under continuous illumination from incandescent lamps (50 µmol photons m^{-2} s^{-1}) according to Wada and Murata, 1989. Δ*crh*R mutant cells, in which the *crhR* gene had been replaced by the spectinomycin resistance gene (*Sp*^{r}) cassette in the genome, were grown under the same conditions as described above with the exception that the culture medium contained spectinomycin at 25 µg ml^{-1} during the pre-cultures. Δ*crhR* mutant cells were then transferred to the above-mentioned BG-11 medium, which did not contain spectinomycin, for the final cell culture for the experiments.

2.2. Composition and preparation of BG-11 medium for culturing *Synechocystis*

For preparation of BG-11 (supplemented with 20mM HEPES –NaOH, pH 7.5) six different stock solutions, each consisting of various micro and macro nutrients were mixed as described below;

Composition of stock 1

Chemical used	Weight (Grams)
Citric acid	0.3
Ferric ammonium citrate	0.3
EDTA	0.05

Dissolved in 100 ml milli Q water and filter sterilized

Composition of stock 2

Chemical used	Weight (Grams)
$NaNO_3$	30
K_2HPO_4	0.7
$MgSO_4.7H_2O$	1.5

Dissolved in one litre milliQ water.

Composition of stock 3

Chemical used	Weight (Grams)
$CaCl_2.2H_2O$	1.9

Dissolved in 100 ml milliQ water.

Composition of stock 4

Chemical used	Weight (Grams)
Na_2CO_3	2

Dissolved in 100 ml milliQ water.

Composition of stock 5

Chemical used	Weight (Grams)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2Mo_4.2H_2O$	0.391
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.\ 6H_2O$	0.049

Dissolved in one litre milliQ water.

Composition of stock 6

Chemical used	Weight (Grams)
HEPES	119.15

Dissolved in 750 ml of milliQ water and adjusted the pH of solution to 7.5 with 2M NaOH. Final volume of the solution was adjusted to one litre with milliQ water.

Except for stock solution 1, all other stock solutions were heat sterilized by autoclaving. Stock solutions were added to milliQ water to prepare 1X - BG11 medium for culturing *Synechocystis.* Volumes of stock solutions required for making 1X –BG11 medium are given in the following table.

Stock solution	1X BG11 medium
Stock 2	50 ml
Stock 3	2 ml

Stock 4	1 ml
Stock 5	1 ml
Stock 6	40 ml
Milli Q water	906 ml

1X - BG11 medium was autoclaved for 20 min at 121^{o}C. Two millilitres of stock solution 1 was added to 1 litre of autoclaved 1X - BG11 medium prior to the inoculation of *Synechocystis* cells. For preparation of solid BG11 media 15 g / L of agar was added to 1000 ml of 1X - BG11 medium

2.3. Antibiotics used in BG11 medium for culturing *Synechocystis* mutants

Name of the mutant	**Name of the Antibiotic used**	**Antibiotic Stock solution**	**Final concentration of Antibiotic**
Δ*crhR*	Spectinomycin	20 mg / ml	20 µg per ml of BG11

2.4. Generation of anti-CrhR antibody

Antibodies against CrhR were raised in rats with His-tagged CrhR of *Synechocystis*, which had been overexpressed in *E. coli*, as antigen. First, we amplified the *crhR* ORF by PCR, with genomic DNA extracted from wild-type cells as template and with the forward primer 5'-GCCATATGACTAATACTTTGACTAGTAC-3'and the reverse primer 5' GCGTCGACTTACTGTTGGCGATCACTATAG-3' and then we purified the product of PCR by electrophoresis on an agarose gel. We eluted the amplified ORF of *crhR* from the gel and inserted it into pET-28a (+) at the *Nde*I and *Sal*I sites to generate pET-CrhR. The amino-terminal His-tagged CrhR protein was expressed in *E. coli* BL 21(DE3) pLysS cells, which had been transformed with pET-CrhR, and it was purified with HIS-Select^{TM} Nickel Affinity gel (P6611; Sigma, St. Luis, Mo, USA) according to the supplier's instruction. The expression of CrhR was induced by the addition of IPTG to a final concentration of 400 mM. Bacterial cells were collected by centrifugation at 10,000 x g for 10 min and pelletted cells

were disrupted with a sonic oscillator **(**model UV2070; probe MS-72; Bandelin Electronic, Berlin, Germany) operated for 10 min at 50% power, with a one-min pulse interval, in 100 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Insoluble materials were removed by centrifugation at 20,000 x *g* for 20 min at 4 ^{o}C. The supernatant was loaded onto a HIS-Select Nickel Affinity column. After the column had been washed with 100 mM Tris-HCl (pH 8.0), 200 mM NaCl and 10 mM imidazole and then with the same buffer that contained 40 mM imidazole, His-CrhR was eluted with a solution of 100 mM Tris-HCl (pH 8.0), 200 mM NaCl and 200 mM imidazole. The purity of each fraction was examined by SDS-PAGE. The fractions that yielded a single band at the expected position on the gel were combined and dialyzed against 5 mM Tris-HCl (pH 8.0). The resultant protein was used by Takara Bio Co. Ltd. (Ohtu, Japan) to raise CrhR-specific antibodies in rats.

Polyclonal Goat anti-rabbit IgG Alkaline phosphatase conjugate was obtained from Sigma Chemical Company (Munich). Antisera against PSII and PSI were kindly provided by Dr.S.Rajagopal (UoH, India) and Dr. Stephan Grenier. (Max Planck, Germany).

2.5. Low temperature treatment

Wild-type and Δ*crhR* cultures which were grown at 34^{o}C until the OD at 730 nm reaches to 0.4, were shifted to a water bath maintained at 24^{o}C, light intensity of 75 µmol photons m^{-2} s^{-1} and constant filter sterilized air supply. *Synechocystis* grows optimally at 34^{o}C. Shift of cultures from 34^{o}C to 24^{o}C would lead to low temperature stress for these organisms.

2.6. Growth curve analysis

To measure the growth rates of *Synechocystis* wild type and mutant strains, each strain was grown at 34^{o}C to an OD of 0.2 at 730 nm and then shifted to respective growth temperatures. Optical density at 730nm was measured for each culture in regular intervals. Usually growth was monitored for every 12 h by measuring the optical density at 730nm.

2.7. Sequence analysis

Gene sequences of *Syechocystis* sp.PCC6803 was obtained from a database of cyanobacterial genomes named 'Cyanobase' at http://www.kazusa.or.jp/cyano/cyano.html.

2.8. Construction of knock-out mutants in *Synechocystis*

2.8.1. Deletional mutagenesis of the *crhR* gene and generation of recombinant plasmids for *crhR* mutagenesis in *Synechocystis*

We generated a Δ*crhR* mutant by replacing the *crhR* gene (*slr0083*) by the Sp^r cassette. A 767-bp upstream and 960-bp downstream flanking regions of open reading frame *slr0083* from the genomic DNA were amplified using primer sets UF (5'AAT CTA GAG TCG ATA TTC CTT GGA TTC GTA TT 3') and UR(5'AAA GGC CTG ACG GTT TAG TGG GCA AAT AAT T 3'); DF (5'AAA GGC CT AAC TCC TCC AGA ACT AAG ACC 3') and DR (5'AAG AGC TCC ATC GAA CCC ATT GAC CTA GAG 3'), respectively. An *Xba*I site and a *Sac*I site (underlined) were created in UF and DR primers, respectively, during primer synthesis. A *Stu*I site (underlined) was created in the primers UR and DF. The PCR fragments, thus generated using UF-UR and DF-DR primer sets, were cloned onto pT7Blue T-A cloning vector separately and named as pTcrh767 and pTcrh960, respectively. The DF-DR fragment released from pTcrh960 plasmid after digestion with *Stu*I and *Sac*I restriction enzymes was ligated to the same sites on pTcrh767. The resultant construct was named $pTcrh^-$. The *Dra*I digested Omega Sp^r cassette was cloned onto the *Stu*I site of $pTcrh^-$ construct by the blunt end ligation. Thus, the final construct, in which the *slr0083* open-reading frame had been replaced by the Omega Sp^r cassette, was used to transform wild-type cells of *Synechocystis*. Genomic DNA extracted from mutant cells was used as the template and UF and DR were used as primers to examine, by PCR, the extent of replacement of the wild-type copy of the chromosome by the mutated copy of the chromosome (Fig.3.7). This

analysis indicated that the wild-type copy of the *crhR* gene had been completely replaced by the mutated copy in Δ*crhR* cells. The resultant mutant was named as Δ*crhR*.

2.9. DNA and RNA analysis

2.9 1. DNA isolation from *Synechocystis*

For isolation of chromosomal DNA *Synechocystis* cells from 35 ml of culture at the late exponential phase (A_{730} of 1.0) were collected, washed with 1 ml of TE buffer and re-suspended in 270 µl STE buffer. The cell suspensions were mixed with 15 µl of chloroform and the mixture was subjected to vigorous vortex mixing for 5 min. Then, 30 µl of lysozyme (20 mg/ml) was added and the cell suspension was incubated for 30 min at 37^{o}C. The lysozyme-treated cells treated with 100 µl of 10% (w/v) SDS and further 10 min after addition of 100 µl of 5 M NaCl. The lysate was deproteinized by chloroform extraction. DNA was precipitated with isopropanol, washed with 70% ethanol and dissolved in sterile water.

2.9.2. PCR analysis

PCR amplification was performed with 0.1 µg of purified *Synechocystis* DNA according to the following programme: 1 cycle of 94^{o}C denaturation for 5 min; 30 cycles at 94^{o}C (30 s), 54^{o}C (1 min), and 72^{o}C (2 min) and an elongation cycle of 10 min at 72^{o}C using Taq polymerase (Sigma Aldrich).

2.9.3. Preparation of cDNAs for DNA microarray analysis

Synechocystis cells that had been exposed to low-temperature stress were killed instantaneously by the addition of 50 ml of a mixture of ice-cold phenol/ethanol (1:20 w/v) to 50 ml of the cell suspension and then total RNA was extracted as described previously (Los et al., 1997). The RNA was treated with DNase I (Nippon Gene, Tokyo, Japan) to remove contaminating DNA. cDNAs, labelled with fluorescent dyes (Cy3 and Cy5; Amersham Pharmacia Biotech), were prepared from 10 µg of total RNA with an RNA fluorescence

Labelling Core kit (M-MLV, version 2.0; Takara Co. Ltd., Kyoto, Japan) according to the manufacturer's instructions.

2.9.4. DNA microarray analysis

Genome-wide analysis of transcript levels was performed with DNA microarrays, as described previously (Kanesaki et al., 2002). We used the *Synechocystis* DNA microarray (CyanoCHIP, Takara Co. Ltd.) that covered 3079 of the 3168 open-reading frames (97% of total genes except transposon-related genes) of the *Synechocystis* genome. Hybridization of the labelled cDNA to DNA microarray was carried out at 65^{o}C for 16 h. After hybridization, the microarrays were rinsed with 2× SSC (1×SSC is 150 mM NaCl and 15 mM sodium citrate) at room temperature. They were washed with 2× SSC at 60^{o}C for 10 min and 0.2× SSC, 0.1% SDS at 60^{o}C for 10 min and then rinsed with distilled water at room temperature for 2 min. Moisture was removed with the air spray prior to analysis with the array scanner (GMS418; Affimetrix, Woburn, MA). Each signal was quantified with the ImaGene ver. 4.0 program (BioDiscovery, Los Angeles, CA, USA). The signal from each gene on the microarray was normalized with reference to the total intensity of signals from all genes with the exception of genes for rRNAs. Then we calculated changes in the level of the mRNA of each gene relative to the total level of mRNA.

2.9.5. Cluster analysis

DNA microarrays of *Synechocystis* (CyanoCHIP ver. 1.6) were purchased from TakaraBio Co. Ltd. (Otsu, Japan). DNA microarray analysis was essentially performed as described in Prakash et al., 2009. Hierarchical clustering of the data was performed using the program Cluster 3.0 (available at http://rana.lbl.gov/EisenSoftware.htm) (Eisen et al., 1998). The averaged data from all microarray analyses (http://www.genome.jp/kegg-bin/get_htext?htext=Exp_DB&hier=1) of down regulated genes with cy5/cy3 ratio ≤ 0.3 were filtered using Microsoft excel. Thus a total of 140 cold repressible genes were used for

isolating thylakoid membrane proteins. Thus the low temperature repressible genes coding for thylakoid membrane proteins in wild-type and Δ*crhR* mutant were log-transformed with log base 2. The metric gene distance was calculated as the Euclidean distance. In cluster analysis, complete linkage clustering was used to organize the genes. The resulting clusters have been visualized using the program TreeView 1.60 (available at http://rana.lbl.gov/EisenSoftware.htm).

2.9.6. Northern blotting analysis

Total RNA was extracted from cells and Northern blotting analysis was performed as described previously (Los et al., 1997). In experiments to examine the stability of transcripts, wild-type and Δ*crhR* cells were incubated in the presence of an inhibitor of transcription, rifampicin, at a final concentration of 100 μg ml^{-1}. At various times after addition of rifampicin to cultures, cells were harvested for extraction of RNA and subsequent Northern blotting analysis. DNA fragment corresponding to the *psaAB* gene and *rnpB* genes were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech) and the resultant conjugates were used as probes. After hybridization, blots were soaked in CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized transcripts were detected with a luminescence image analyzer (LAS-1000; Fuji-Photo Film, Tokyo, Japan).

In experiments to examine the stability of transcripts, wild-type and Δ*crhR* cells were incubated in the presence of an inhibitor of transcription, rifampicin, at a final concentration of 100 μg ml^{-1} and DNA fragment corresponding to the *psaAB* gene and *rnpB* genes were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech) and the resultant conjugates were used as probes. Northern analysis was carried out as described above.

2.10. Pigment analysis of *Synechocystis* cells

2.10.1. Determination of chlorophyll *a* concentrations

The content of chlorophyll *a* was estimated according to Arnon et al., (1974). Cell suspension of 1 ml was centrifuged at 10.000 x g for 10 min at 4^{o}C and the pellet was extracted with 90% (v/v) methanol for 1 h at -20^{o}C, followed by centrifugation at 10.000 x g for 10 min at 4^{o}C. The chlorophyll *a* content was calculated from the absorbance of the methanol extract at 652 and 665 nm using the equation:

Chlorophyll (mg/ml) = 16.82 x A665 - 9.28 x A652.

2.10.2. Determination of C-PC concentrations

The content of PBP was estimated according to Grossman et al., (1993). The cell samples were heated at 75^{o}C for 10 min and the PC content was determined according to the following equation:

PC (mg/ml) = [A620 nm. A750 (unheated)] - [A620 nm. A750 (heated)].

2.10.3. Absorption spectroscopy

Absorption spectra of cell suspensions were recorded with a UV-3000 (Shimadzu, Japan) spectrophotometer. The cell densities were adjusted to OD at 730 nm. The absorption spectra of cell suspensions were scanned in the visible region from 400 to 750 nm. All these absorption spectra were taken at room temperature and were not corrected for spectral sensitivity.

2.10.4. Room temperature and 77K fluorescence spectroscopy

The fluorescence emission spectra were recorded from 600 to 750 nm at room temperature and low temperature (77K) using a Perklin Elmer spectro-fluorimeter (model: LS55, USA) for both cell suspensions and fractions obtained after sucrose density gradient ultracentrifugation, respectively. The excitation and emission slit width were set at 10 and 5 nm, respectively. The excitation wavelength was either 436-nm primarily absorbed by

chlorophyll *a* or 580-nm which preferentially excites the PBS. The samples had ~ 0.4 O.D at 730 nm. The spectra were corrected for the sensitivity of the photomultiplier and normalized to the maximum of PSII emission around 650 nm.

2.11. Measurement of photosynthetic electron transport rates

Photosynthetic oxygen evolution was measured in 1ml of cell suspension (OD_{730} of ~ 1, about 5 µg / ml chlorophyll), using oxygen electrode (Oxygraph plus, Hansatech Instruments Ltd., Norfolk, England). The cells were cultivatedphotoautotrophically under aeration at 70 µmol photons m^{-2} s^{-1} light. PSII activity was determined in the presence of 1.0 mM para-phenyl-benzoquinone (PPBQ) (Sigma) at 1000 µE m^{-2} s^{-1}. PSII activity was determined as a difference of net oxygen evolution in light and oxygen consumption in the dark.

2.12. Chlorophyll fluorescence induction curves and Fo′measurements

Slow fluorescence induction curves were recorded using Dual PAM100 chlorophyll fluorescence photosynthesis analyzer (Heinz Walz, Germany) (Fig.2.1) for *Synechocystis* wild-type and Δ*crhR* mutant cells at 34^{o}C and also after incubation of cells at 24^{o}C for 72 h. Fluorescence was measured using the DUAL DB head with the instrument operated in the fast kinetics mode. Actinic illumination was provided by two arrays of 635-nm LEDs illuminating the cell cultures. State transition experiments were performed using cultures according to established protocols (Haldrup et al., 2001). Preferential PSII excitation was provided by illumination with red light at an intensity of 37 µmol photons m^{-2} s^{-1} provided by a KL1500 lamp equipped with a 650-nm interference filter, and excitation of PSI was achieved using far-red light from an LED light source (Heinz Walz; 102-FR) applied for 2 min simultaneously with red light. Periods of red, far-red light conditions were used alternately in several cycles, and the Fm level in State 1(Fm^1) and State 2 (Fm^2) was determined at the end of each cycle by the application of a saturating light pulse (4000 µmol photons m^{-2} s^{-1}) described above. Reduction state of the PQ pool was assessed following the

Fig.2.1. Plant efficiency analyzer (PEA); B. Pulse Amplitude modulation fluorometry (PAM). PEA and PAM were used for measuring Chl *a* fluorescence transients.

post illumination transient increase of chlorophyll fluorescence at the Fo′ level (Asada et al., 1993; Mano et al., 1995).

2.13. Chlorophyll *a* fluorescence measurements using handy PEA (plant efficiency analyser)

Chlorophyll *a* fluorescence fast and slow induction curves were measured using a plant efficiency analyzer (PEA), Hansatech, King's Lynn, Norfolk, UK (Fig.2.1). The cultures were dark adapted for 20 min and then excited by an array of three light-emitting diodes peaking at 650 nm at a photon flux density of 3000 μmol photons m^{-2} s^{-1}. It detects fluorescence at wavelengths above 700 nm (50% transmission at 720 nm) and records it continuously from 10 μs to 5 min. The fast fluorescence transients (OJIP) and slow transients (SMT) were measuredfrom wild-type and Δ*crhR* mutant cultures both at 34^{o}C and 24^{o}C according to Stamatakis et al., 2007.

2.14. Preparation of thylakoid membranes

Synechocystis cells were harvested in the mid-log growth phase and pelleted by centrifugation at 5000 g for 10 min at 4^{o}C. Cells were resuspended in 500 μl of 50mM Tris-HCl, pH 8.0 buffer and disrupted using 500 mg of 0.5 mm glass beads (acid washed, Sigma Aldrich). The cells were disrupted by vortex mixing for 5 min on ice, with 30sec intervals between each minute of vortex mixing. Glass beads, unbroken cells, and cell debries were removed by centrifugation for 5 min at 4^{o}C and 5,000 g. The homogenate was transferred to a new microfuge tube and subjected to centrifugation at 18,500 g for 40 min. The membrane pellet was resuspended in 100 μl of 50 mM Tris-HCl, pH 8.0 containing 25% w/v glycerol.

2.15. Separation of thylakoid membrane supercomplexs by sucrose density gradient centrifugation

Thylakoid membrane supercomplexes were isolated according to Kargul et al., (2003) by sucrose density gradient ultracentrifugation (SDGUC) with some modifications. Thylakoid

membranes (1mg Chl/ml) were solubilized withn-dodecyl β-D Maltoside (DDM) (Sigma) at a final concentration of 0.9% (w/v); this sample was stirred slowly for 20 min at 4^{o}C and centrifuged at 48,400x g. 0.2 ml supernatant was loaded on a 5 ml of 0.25-2.0 M sucrose density gradient, containing 5 mM Tricine-NaOH pH 8.0, 0.5 M sucrose, 0.5 M betaine and 0.05% DDM (w/v). After centrifugation at 180,000 x g for 16 h, 2 fractions were collected majorly. The fraction 1 (F1) is enriched with PSII and PSI supercomplexes and F2 is enriched with PSI supercomplexes. The isolated fractions were characterized by 77K fluorescence emission spectroscopy and BN-PAGE analysis.

2.16. Non-Denaturing gel electrophoresis

2.16.1. Blue native PAGE

Supramolecular photosynthetic complexes were separated and analysed by BN-PAGE according to Schagger et al., 1991 with minor modifications.Isolated thylakoids were resuspended in 60 µl of ACA buffer (50 mM Imidazole pH 7.0, 500 mM 6-aminocaproinc acid, 1Mm EDTA) (end concentration 1 µgµl^{-1} chlorophyll) and solubilized by addition of 10 µl of n-dodecyl β-D Maltoside (DDM)(Sigma) (10% (w/v) in H_2O) for 15 min at 4^{o}C under constant rotation. Unsolubilized membranes were pelleted out by ultracentrifugation at 45,000 x g for 30 min at 4^{o}C. The supernatant were mixed with 5 µl of sample buffer (50 mM Bis-Tris, pH 7.0, 500 mM 6-aminocaproic acid, 5% coomassie brilliant blue G (CBB-G)). The samples were then loaded onto an 8% gel and ran at 100 V. After entering the sample toresolving gel, electrophoresis was continued to run at 500 V. When the dye front had reached half the gel distance, cathode buffer (7.5 mM imidazole, pH 7.0, 50 mM tricine containing 0.02% CBB-G) was replaced by the same buffer without staining reagent (CBB-G). All solutions were pre-cooled to 4^{o}C; electrophoresis was performed at 10^{o}C.

2.16.2. Green gel electrophoresis for resolving pigment protein complexes

Green gels were performed according to Laemmli (1970) without SDS and DTT (Dithiothreitol). Both the separating (10%) and stacking gels (5%) did not contain any detergent. Electrophoresis buffer consisted of 0.01% Lithium dodecyl sulphate (LDS). Sample preparation was done by solubilising thylakoid membrane by mixing equal volume of 2X sample buffer containing 125 mM Tris-HCl (pH 6.8), 20% glycerol and 10% w/v DDM for 25 min at 4^{o}C under constant rotation. Unsolubilized membranes were pelleted out by ultracentrifugation at 45,000 x g for 30 min at 4^{o}C. Supernatant containing solubilised thylakoid membranes equivalent to 30 µg of Chl was loaded on each lane of the 8% SDS-PAGE gel (4% stacking gel, 8% resolving gel). Green gels were run at 4^{o}C, at a constant voltage of 90-100 V for 1.5 h. The pigment-protein complexes were obtained similarto Delepelaire and Chua, 1979.

2.17. Epifluorescence microscopy

Stock solutions of SYTOX Green (Molecular Probes) fluorescent dye from the ViaGram Red Bacterial Gram Stain and Viability Kit (Molecular Probes) were prepared according to the protocol provided by Molecular Probes. SYTOX dye discriminates live cells from dead cells on the basis of plasma membrane integrity. Two nucleic acid stains were used for viability determination. Bacteria with intact cell membrane stains fluorescent blue with 4',6-diamidino-2-phenylindole(DAPI), and bacteria with damage membrane stain fluorescent green with Sytox Green nucleic acid stain. Light exposure which causes the dye to fade was minimized using aluminum foil wrapped microfuge tube to incubate the samples and to store the dye stocks. Because of the pH sensitivity of the dye all samples were resuspended in BG-11 medium, pH 7.5 prior to mixing with SYTOX Green solution. Samples were added to slides and visualized by fluorescence microscopy using Olympus BX61 fluorescence microscopy.

Dead cells were visualized with SYTOX Green dye under fluorescence microscopy and appeared in green colour. Auto-fluorescence of pigments can be visualized in red.

2.18. Transmission electron microscopy

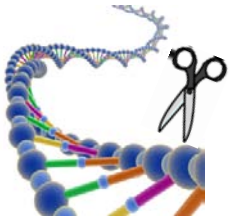
Samples were fixed in 2.5 % - 3% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4^{o}C and post fixed in 2% aqueous Osmium tetroxide in the buffer for 2 h. Dehydrated in series of graded alcohols to filtrate and embedded in araldite 6005 resin or spur resin (Spurr 1969). Ultrathin (50-70 nm) sections were made with a glass knife on ultramicrotome (Leica ulta cut D ON UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead citrate (Bozzola and Russel, 1998). Viewed under TEM (Model; Hitachi, H-7500, from JAPAN) at required magnification as per the standard procedures at RUSKA Lab, College of Veterinary Sciences, SVVU, Rajendranagar, India.

2.19. Immunoblotting analysis

To investigate the levels of PSI and PSII proteins in wild-type and Δ*crhR* cells, the blots were probed with PsaB, PsaA and D1 antibodies (1/2500 dilution) (Agrisera, Sweden) for detection of these proteins of thylakoid membranes in both wild-type and Δ*crhR* cells.

Mutagenesis of the gene coding for RNA helicase, *crhR* in *Synechocystis* sp. PCC 6803 and phenotype characterization of the mutant

Chapter 3



CrhR gene deletion

3.1. Introduction

The main aim of this objective is to generate Δ*crhR* mutant by deletional inactivation method and characterize the Δ*crhR* mutant during low temperature conditions.

3.2. Results and discussion

3.2.1. Establishment of culture growth conditions and temperature treatment

Synechocystis sp. PCC 6803 grows optimally at 34^{o}C under continuous illumination of 75 µ E $m^{-2}s^{-1}$ and 1% CO_2. *Synechocystis* cultures which were previously acclimatized to 34^{o}C for several rounds of culturing, when shifted to 24^{o}C, 20^{o}C, 15^{o}C and 10^{o}C exhibited reduction in growth rate (Fig.3.1). At 10^{o}C and 15^{o}C cell division was completely arrested. At 20^{o}C growth rate was much strongly reduced when compared to the cells grown at 24^{o}C. Based on the growth profiles of *Synechocystis* cultures we have chosen 24^{o}C as low temperature treatment and 34^{o}C as optimal growth temperature for further experiments.

3.2.2. Gene coding for RNA helicase

Kaneko et al. determined the entire nucleotide sequence of the genome of *Synechocystis* together with the entire sequences of four plasmids harbored by *Synechocystis* (Kaneko et al., 1996; Kaneko et al., 2003). This is particularly useful as basic information, which can be exploited for genome-wide studies of gene expression. About 10 years ago, Takara Bio Co. (Ohtu, Japan) initiated the production of genome-wide DNA microarrays for the analysis of gene expression in *Synechocystis*. The DNA microarray covers 3,079 (97%) of the 3,165 genes on the chromosome of *Synechocystis*, excluding 99 genes for transposases. It does not carry the genes from the four plasmids as well. The original results of analysis of patterns of gene expression in this cyanobacterium can be found in the KEGG expression database (Lists of experimental data are available at http://www.genome.jp/kegg/expression/).

The database of genome sequences available at http://www.kazusa.or.jp suggests that *Synechocystis* contains only one gene, *crhR* (open reading frame, *slr0083*), for RNA helicase.

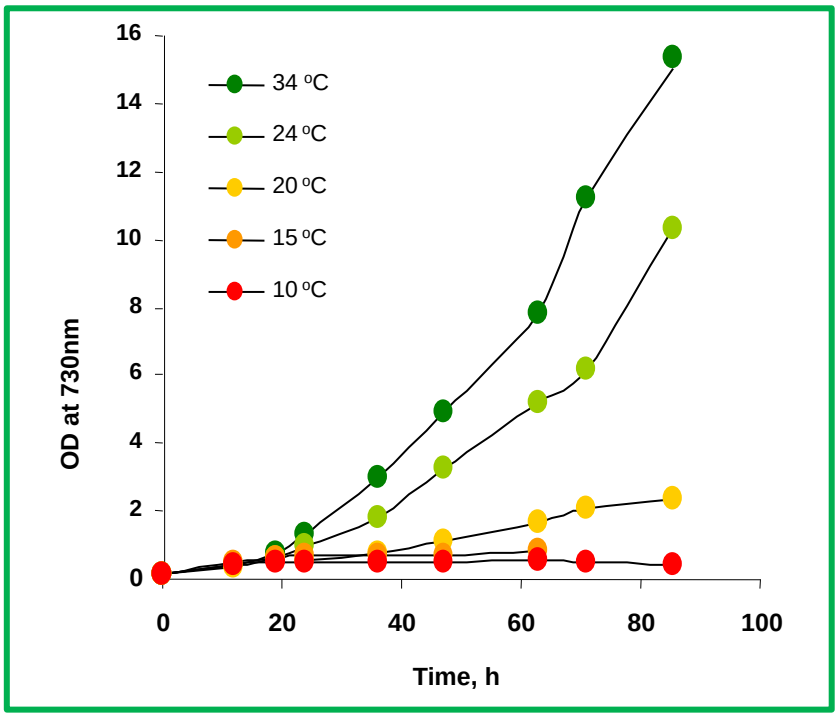


Fig.3.1. Effect of low temperature on growth of *Synechocystis* sp. PCC 6803. For growth at 34^{o}C, *Synechocystis* cultures were inoculated in fresh BG11 medium to 0.1 OD at 730nm and then measured optical density at regular intervals. For monitoring growth at low temperature, cultures were initially allowed to grow at 34^{o}C for 16 h and then shifted to water bath maintained at 24, 20, 15 and 10^{o}C. Optical density at 730 nm was measured at regular intervals until 82 h of low temperature incubation.

As mentioned earlier the *crhR* gene for RNA helicase is cold inducible whose expression is highly upregulated upon low-temperature stress in *Synechocystis* (Suzuki et al., 2001; Los et al., 2008; Prakash et al., 2010). Heterologous expression of the *crhR* gene in *E. coli* and subsequent biochemical characterization of the expressed protein demonstrated that this protein catalyzed both ATP-dependent unwinding of secondary structures of RNA and annealing of complementary RNA strands (Chamot et al., 2005). Although CrhR, *in vitro* is active in unwinding, annealing and exchanging RNA strandsits function during acclimatization of *Synechocystis* cells to low temperature has not been demonstrated. Fig.3.2 shows the map position of *slr0083* (ORF encoding RNA helicase, CrhR) and the open reading frames located either side of *slr0083*.

3.2.3. *crhR* gene expression is transiently upregulated upon a downward shift in temperature

Earlier reports suggested that expression of the *crhR* gene is induced under low-temperature, salt and hyperosmotic stress in *Synechocystis* (Vinnemeier and Hagemann, 1999; Suzuki et al., 2001; Kanesaki et al., 2002). Figure 3.3 shows changes in the level of *crhR* mRNA during exposure of *Synechocystis* cells, which were grown at 34^{o}C and then shifted to 24^{o}C for designated period of time. A low level of *crhR* mRNA was detected before the exposure, indicating that this gene was constitutively expressed in *Synechocystis.* The downward shift in temperature (24^{o}C) increased transiently the level of *crhR* transcript with the maximum attained at 30 min (Fig.3.3).

3.2.4. CrhR protein accumulates after a downward shift in temperature

To examine whether the level of CrhR protein reflects the transient increase in the level of *crhR* mRNA during incubation at low temperature, we performed western blotting analysis to monitor the changes in the CrhR level during exposure of *Synechocystis* cells to 24^{o}C (Fig.3.4). The anti-CrhR antibody raised in rats detected CrhR at a molecular mass of

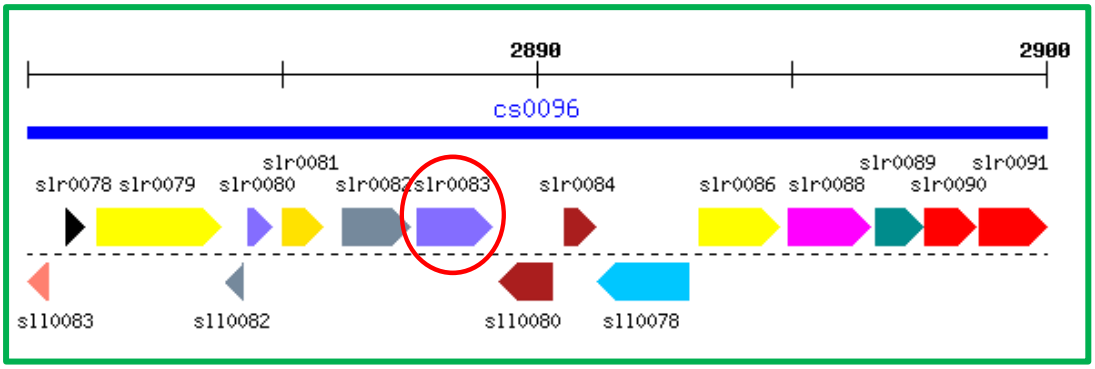


Fig.3.2. Representation of the location of the *crhR* gene in the genome of *Synechocystis.* Red circle represents the open reading frame *slr0083* with its adjacent ORFs, *slr0082* and *sll0080.* The nomenclature for *slr0083* ORF is s: represents *Synechocystis*, l: represents the length of the orf (more than 100 codons in length is long), r: orientation of the ORF which is towards right. Adapted from Cyanobase.

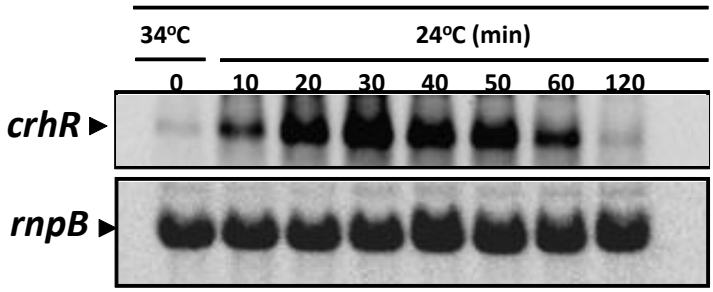


Fig.3.3. Northern-blotting analysis of changes in the expression of the *crhR* gene upon downward shift of temperature in wild-type cells of *Synechocystis.* Total RNA was extracted from wild-type cells that had been grown at 34°C for 16 h or had been grown at 34°C for 16 h and then incubated at 24°C for 10, 20, 30, 40, 50, 60 and 120 min. Aliquots (20 μg) of the extracted RNA were electrophoresed on 1.2% agarose gels that contained 1.4 M formaldehyde.

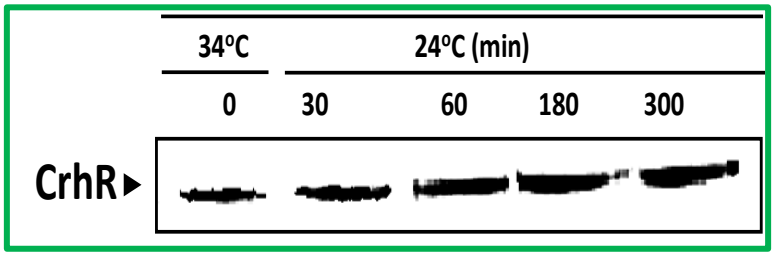


Fig.3.4. Changes in the level of CrhR in wild-type and Δ*crhR* cells after a downward shift in temperature. Soluble proteins were extracted from wild-type and Δ*crhR* cells that had been grown at 34°C and then incubated at 24°C for 30, 60, 180, and 300 min. Samples equivalent to 25 μg of proteins were loaded in each well. Experiment was performed twice and the results are presented as a mean of the two independent experiments.

approximately 52 kDa (Fig.3.4). Fig.3.4 shows that CrhR was present at a certain level before the downward shift in temperature. Then the level of CrhR gradually increased during incubation at 24^{o}C and reached a maximum level that was 3.5 times as high as the original after incubation at 24^{o}C for 180 min (Fig.3.5). Quantitative analysis of *crhR* transcript and protein levels indicated that *Synechocystis* cells induced the expression of the *crhR* gene for RNA helicase and accumulated CrhR protein when the temperature was shifted downward by 10^{o}C from optimal growth temperature, 34^{o}C (Fig.3.5).

Earlier studies using DNA microarrays demonstrated that almost all of the low temperature-inducible genes in *Synechocystis* are under the control of the sensory kinase, Hik33, except the *crhR* gene (Suzuki et al., 2001). This indicates that the low temperature-inducible expression of the *crhR* gene might be regulated by a mechanism other than the two-component signal transduction.

3.2.5. Generation of mutant Δ*crhR*

3.2.5.1. Deletional mutagenesis of the *crhR* gene

As described in Materials and methods, a Δ*crhR* mutant was generated by replacing the *crhR* gene (*slr0083*) by the *Sp*^{r} cassette. The resultant construct was named pTcrh^{-}. The *Dra*I digested Omega *Sp*^{r} cassette was cloned onto the *Stu*I site of pTcrh^{-} construct by the blunt end ligation (Fig.3.6). Thus, the final construct, in which the *slr0083* open-reading frame had been replaced by the Omega *Sp*^{r} cassette, was used to transform wild-type cells of *Synechocystis* (Fig.3.6).

3.2.5.2. Segregation of the mutants Δ*crhR*

As described in Materials and Methods, after several rounds of culturing of mutant cells in the presence of various concentrations of spectinomycin, the genomic DNA was isolated to check the extent of replacement of wild-type copies of the *crhR* with the mutated copies. With the genomic DNA from wild-type cells as a template, a fragment of 3.2 kb that

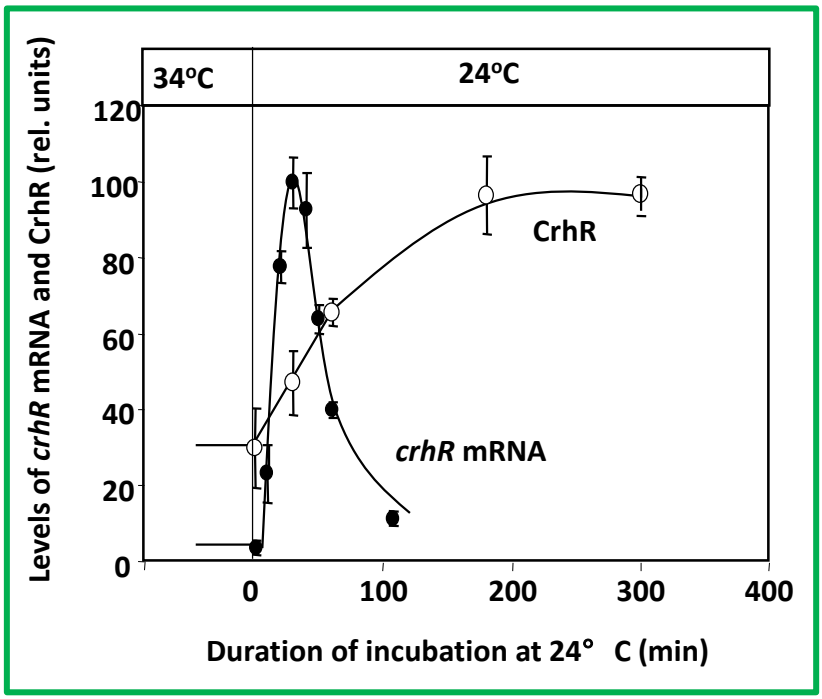


Fig.3.5. Quantitative expression of *crhR* mRNA and CrhR protein upon the downward shift in temperature. Increase in the level of CrhR during incubation at 24°C is indicated by open circles. Transient increase in the level of *crhR* transcript attained at 30 min during the incubation at 24°C is indicated by solid circles.

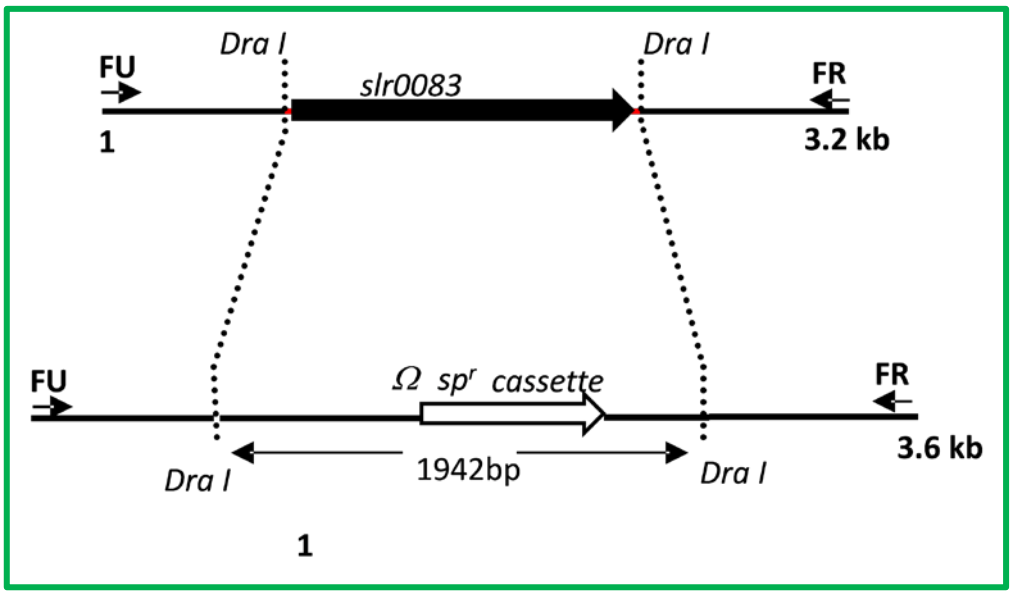


Fig.3.6. Strategy for disruption of the *crhR*gene in the genome of *Synechocystis*sp. PCC 6803. The *crhR* (*slr0083*) gene and the spectinomycin-resistance gene (Sp^r) cassette are shown in the filled and open arrows, respectively. Thick arrows indicate UF and DR primers used for PCR amplification of the wild-type copy of the *crhR* gene and that of the sp^r cassette.

corresponded to the *slr0083* ORF and its upstream and downstream flanking regions was amplified by PCR. In contrast, with the same set of primers, 3.2 kb PCR fragment was not amplified, when the genomic DNA of Δ*crhR* cells was used as template. Instead, a 3.6 Kb DNA fragment, corresponding to the upstream and downstream flanking regions of *crhR* ORF, with the spectinomycin gene cassette was amplified (Fig.3.7). These observations suggest that the *slr0083* ORF has been completely inactivated by replacement of spectinomycin gene cassette in *Synechocystis.*

3.2.6. Characterization of RNA helicase mutation in *Synechocystis*

3.2.6.1. Physiological analysis of helicase knock-out

Helicase deficient strains and wild-type were grown under standard conditions till A_{730} = 0.4 and then transferred to low temperature stress at 24^{o}C and phenotype was monitored for 72 h to analyse the acclimation of wild-type and Δ*crhR* to low temperature conditions. Physiological characterization of the Δ*crhR* mutant did not show any significant difference in the growth when compared to wild-type cells under optimum conditions. At 34^{o}C these two types of cells revealed similar profiles of growth (Fig.3.8A). However at 24^{o}C, growth of Δ*crhR* cells was much slower than that of wild-type cells (Fig.3.8B), indicating that the low temperature-induced expression of the *crhR* gene and synthesis of CrhR were important for *Synechocystis* cells to survive at low temperature.

Fig.3.9 shows the wild-type and Δ*crhR* cultures grown in tubes under continuous illumination of 70 µmol photons m^{-2} s^{-1}. At 34^{o}C, no difference was observed in the growth profiles between wild-type and Δ*crhR* cells. The wild-type cultures looked similar to that of Δ*crhR* cultures and no distinguishable difference in the colour of the cultures was observed (Fig.3.9A). However, incubation of Δ*crhR* cells at 24^{o}C for 48h and 72 h resulted in a bleached phenotype when compared to wild-type cells (Fig.3.9B and C). This bleached phenotype could be either due to reduced growth rate and /or loss of pigment content in the

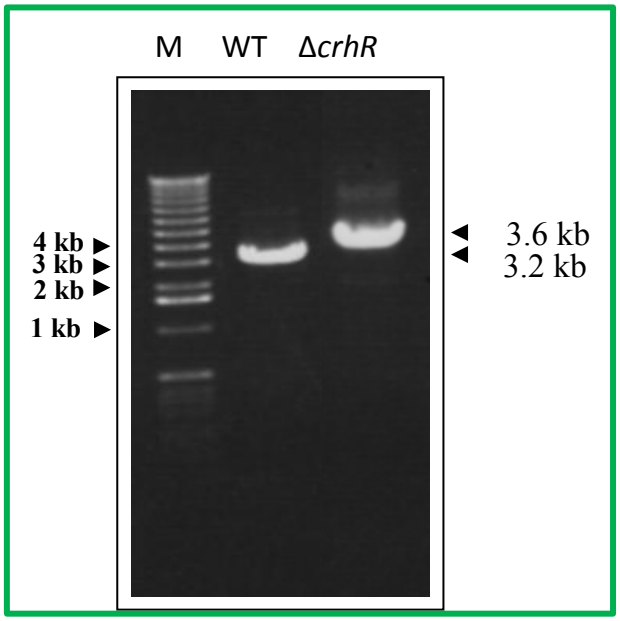


Fig.3.7. Genomic PCR analysis. WT: Genomic DNA extracted from wild-type cells was used as the template with UF and DR primers to examine the extent of replacement of wildtype copies of the *crhR* with the disrupted copies of *crhR*. Δ*crhR*: Genomic DNA extracted from Δ*crhR* cells was used as template. M represents 1-kb DNA ladder.

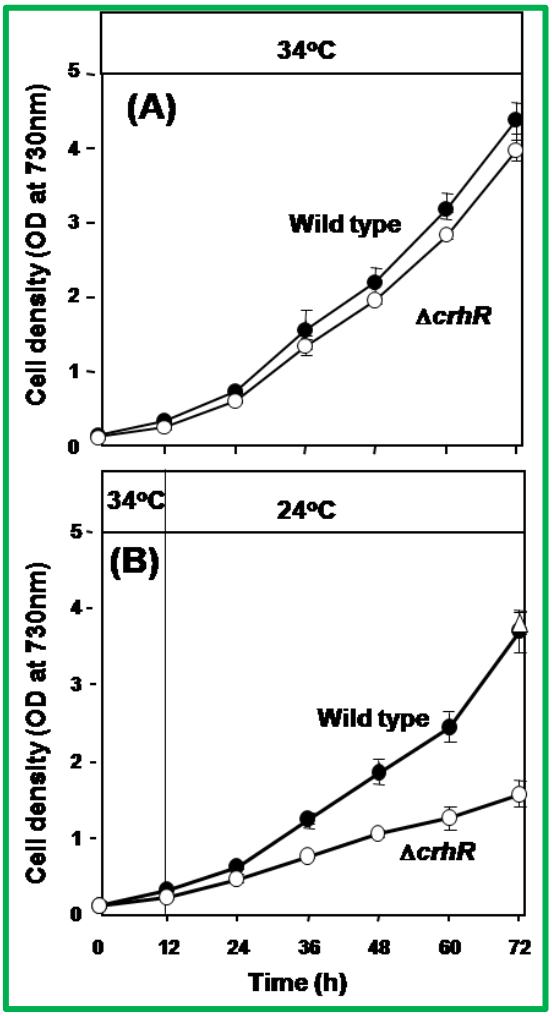


Fig. 3.8. Mutation of *crhR* gene affected growth profiles at low temperature. Wild-type cells (●), Δ*crhR* mutant cells (○) were grown photoautotrophically at 70 μmol photons $m^{-2}s^{-1}$ at 34 ^{o}C (A) or 24^{o}C (B). Cell growth was monitored by measuring the apparent absorbance at 730 nm. Similar results were obtained in three independent experiments, and the data represented as mean ±SD.

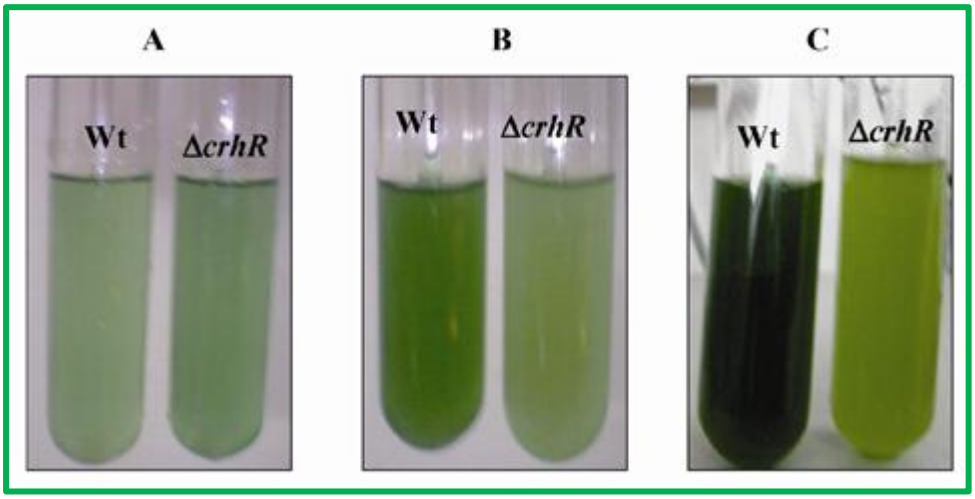


Fig. 3.9. Effect of *crhR* mutation on the growth of *Synechocystis* cells at low temperature. (A) Wild type and Δ*crhR* mutant cells were grown photoautotrophically at 34^{o}C for 16 h. (B) Wild-type and Δ*crhR* mutant cells, which had been grown at 34^{o}C for 16 h, were incubated at 24^{o}C under continuous illumination of 70 µmol photons m^{-2} s^{-1} for 48 h. (C) Wild-type and Δ*crhR* mutant cells, which had been grown at 34^{o}C for 16 h, were incubated at 24^{o}C for 72 h. WT, wild-type cells; Δ*crhR*,Δ*crhR* mutant cells.

Δ*crhR* mutant cells at low temperature, suggesting an important functional role of CrhR during low temperature acclimation.

3.2.6.2. Effect of low temperature on cell viability and cell division in wild-type and Δ*crhR*

Cyanobacterial cells when examined under fluorescence microscope appear as bright red cells due to the auto-fluorescence emitted from chlorophyll *a* (Miyagishima et al., 2005). To check the viability of the wild-type and Δ*crhR* after low temperature stress at 24ºC cells were stained with Sytox Green Dye. As we observed a bleached phenotype of Δ*crhR* cells during low temperature incubation, the wild type and Δ*crhR* cells were examined under fluorescence microscopy for the extent of auto-fluorescence emitted by the cells, which can be related to changes if any in the pigment content of the cell. Both wild type and Δ*crhR* cells which were grown at 34^{o}C for 16 h (here after WT-34^{o}C cells and Δ*crhR*-34^{o}C cells respectively) appeared as bright red fluorescent cells (Fig.3.10A and B). When wild-type and Δ*crhR* cells which were grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h (here after WT-24^{o}C-cells and Δ*crhR*-24^{o}C cells) there was no significant difference in the appearance between WT-24^{o}C cells and WT-34^{o}C cells(Fig.3.10C). In contrast, Δ*crhR*-24^{o}C cells emitted reduced level of auto-fluorescence and appeared as dull red-fluorescent cells when compared to the WT-24^{o}C cells, indicating relatively low chlorophyll *a* content in the Δ*crhR*-24^{o}C cells when compared to the WT-24^{o}C cells (Compare Fig.3.10C and D). The SYTOX Green stain permeabilizes through the damaged membranes and stains the dead cells and appeared green under fluorescence microscope while the live cells doesn't take up the stain (Fig.3.10). As seen in the Figure 3.10, almost all the wild-type cells were found to be viable before low temperature treatment. Very few cells were found to be nonviable and stained green after low temperature treatment which indicates that it is a usual phenomenon in both wild-type and Δ*crhR* cells. Bilobed nucleoids with undivided cells can be clearly visualized under

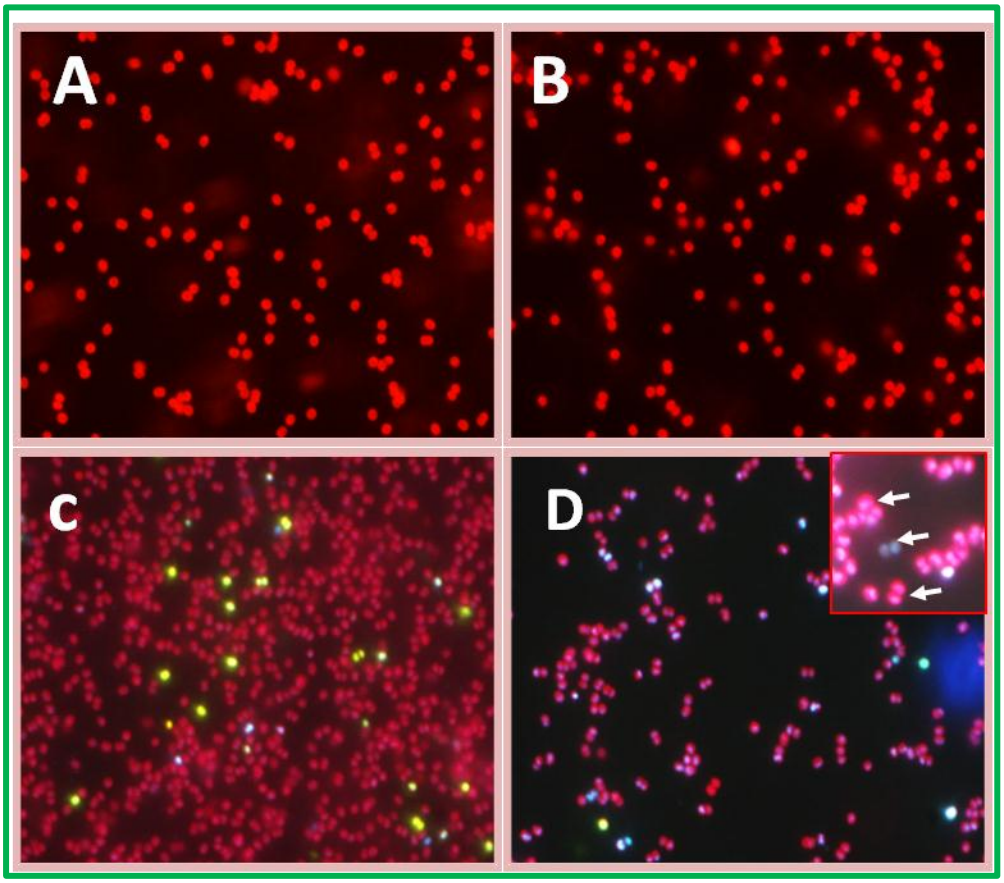


Fig.3.10. Effect of *crhR* mutation on the survival of *Synechocystis* cells at low temperature.Wild- type and Δ*crhR* cells were grown at 34^{o}C for 16 h (34^{o}C cells). The 34^{o}C cells were further incubated at 24^{o}C for 72 h (24^{o}C cells). Both types of cells were stained with SYTOX Green dye (molecular probes) and observed with a fluorescence microscope (Olympus SX61). (A) WT-34^{o}C cells; (B) Δ*crhR* 34^{o}C-cells; (C) WT-24^{o}C cells; (D) Δ*crhR*-24^{o}C cells. A few bilobed nucleoids are indicated by the arrows in the inset of (D). Cells were observed under 60X magnification.

fluorescence microscope when stained with the nucleoid stain DAPI. When Δ*crhR*-24^{o}C cells were observed under microscopy relatively more number of cells appeared as bilobed nucleoids when compared to WT-24^{o}C cells indicating that there was a slower rate of cell division of the Δ*crhR* cells when compared to the wild-type cells after low temperature treatment (Fig.3.10D Inset). This probably suggests that *CrhR* might have an important role in cell division during low temperature acclimation of *Synechocystis*.

3.2.6.3. Effect of low temperature on ultrastructure of the cell

As we observed a significant difference in the shape of Δ*crhR* cells at low temperature as compared to wild type cells, we further examined the changes in the ultrastructure of *Synechocystis* wild-type and Δ*crhR* cells by Transmission electron microscopy (TEM). We monitored the changes in thylakoid membrane architecture in wild-type and Δ*crhR* cells grown at 34^{o}C. As can be seen in Fig.3.11, WT-34^{o}C cells and Δ*crhR*-34^{o}C cells looked almost alike with similar kind of thylakoid membrane organization (Fig.3.11A and B). Therefore, depletion of *crhR* did not result in reduction of thylakoid membranes under optimum growth conditions.

Shift of the cultures to low temperature resulted in an alteration in the thylakoid membrane architecture was observed in the wild-type (Fig.3.11C). The thylakoid membranes seemed to be less well orgnaized. The cells appeared to be small in size and the thylakoid membrane architecture was found to be modified. Probably such ultrastructual alterations could be an adaptative response to low temperature acclimation (Fig.3.11C). However,Δ*crhR*-24^{o}C cells did not show any changes in the organization of thylakoid membrane, as observed in WT-24^{o}C cells (compare Fig.3.11C and D). In the Δ*crhR*-24^{o}C cells the thylakoid membranes lacked the beaded appearance indicating association of less number of phycobilisomes with the photosynthetic membranes (Fig.3.11D). The Δ*crhR*-24^{o}C cells appeared to be large in size when compared to the Δ*crhR*-34^{o}C cells. This

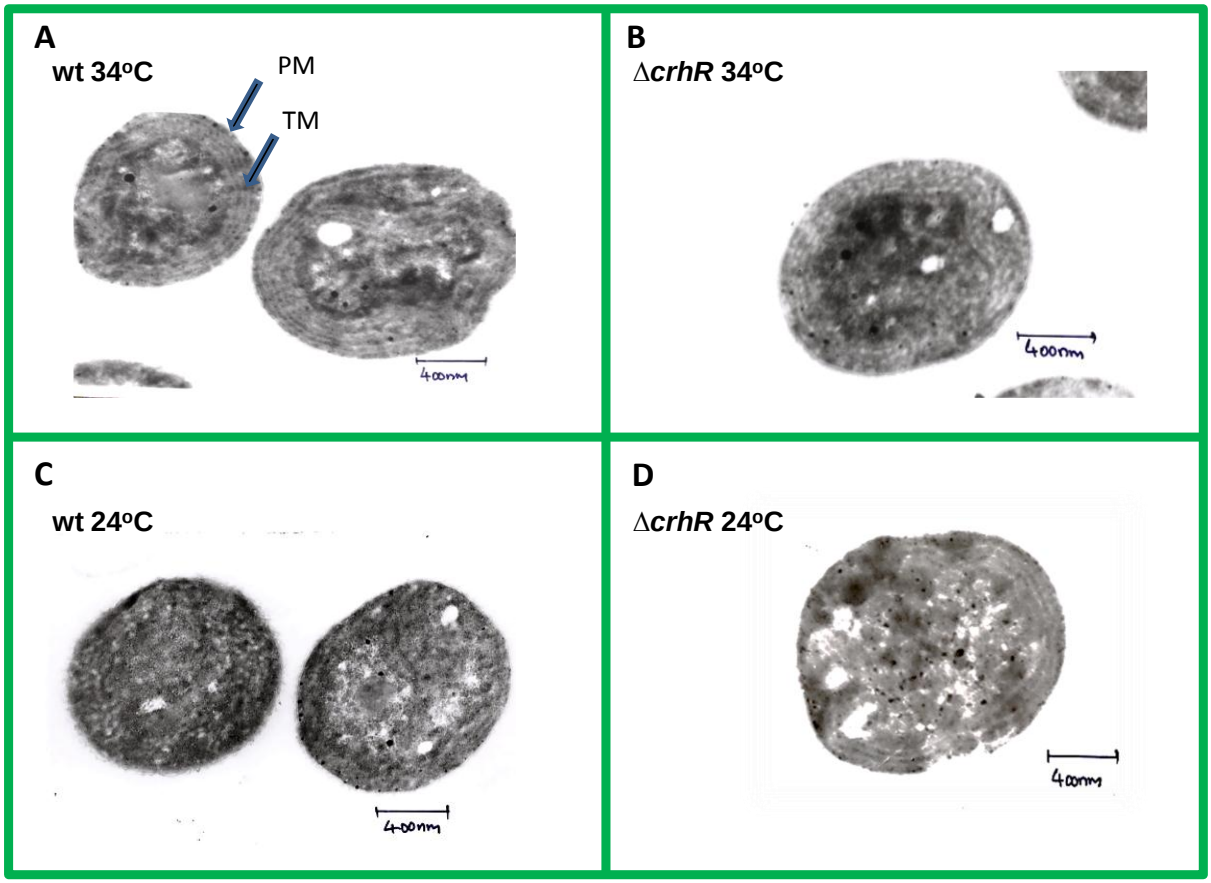


Fig.3.11. Transmission electron micrographs of wild-type and Δ*crhR* cellsgrown at 34°C and shifted to 24°C for 72h. (A) WT-34°C-cells; (B) Δ*crhR*-34°C cells; (C)WT-24°C cells; (D)Δ*crhR*-24°C cells.

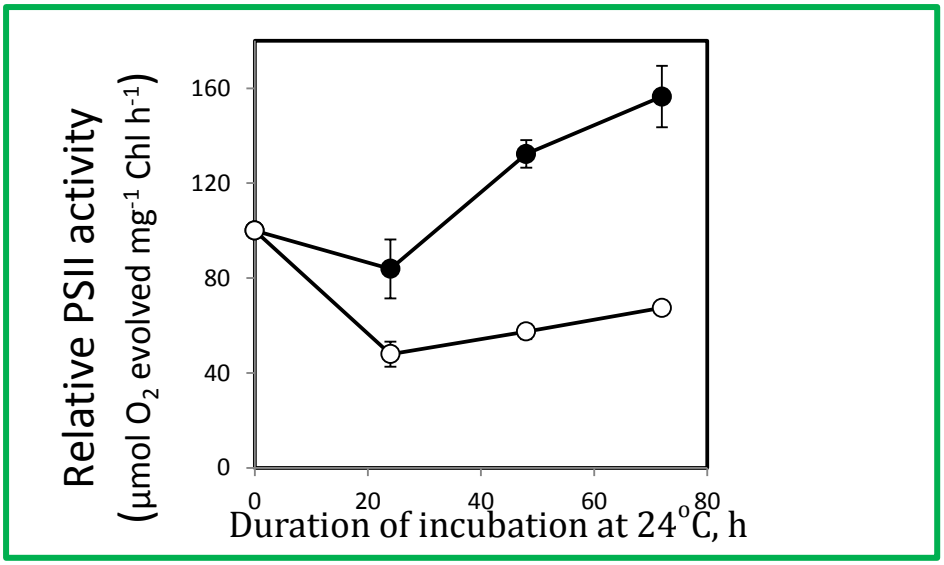


Fig.3.12. PSII activity of wild- type and Δ*crhR* cells which had been grown at 34°C and then shifted to 24°C for 22, 44 and 72 h. Rate of photosynthetic electron transport was measured in terms of oxygen evolution using an oxygen electrode. PSII activity in wild type cells (-●-) in Δ*crhR* cells (-○-).

suggests that the cells depleted of *crhR* failed to alter its thylakoid membrane architecture and cell size at low temperature.

3.2.6.4. Impact of deletion of *crhR* gene on PSII activity

To further characterize the effect of mutation, we measured the PSII activity. At 34^{o}C there was no significant difference in the PSII activity (from water to PBQ) between wild type and Δ*crhR* cells (Fig.3.12). Upon shift of cultures to low temperature, a significant drop in the PSII activity is seen in the wild-type. This drop was much prominent in the Δ*crhR*. However, a gradual increase in the PSII activity was observed during incubation of wild type cells at 24^{o}C (Fig.3.12). This is in agreement with that of the previous studies on low temperature stress in *Synechococcus* PCC 7002, in which an increase in the photosynthetic activity was reported due to incubation of cells at low temperature (Sakamoto and Bryant, 1998). In contrast, low temperature induced enhancement in the PSII activity was not observed in the Δ*crhR* cells.

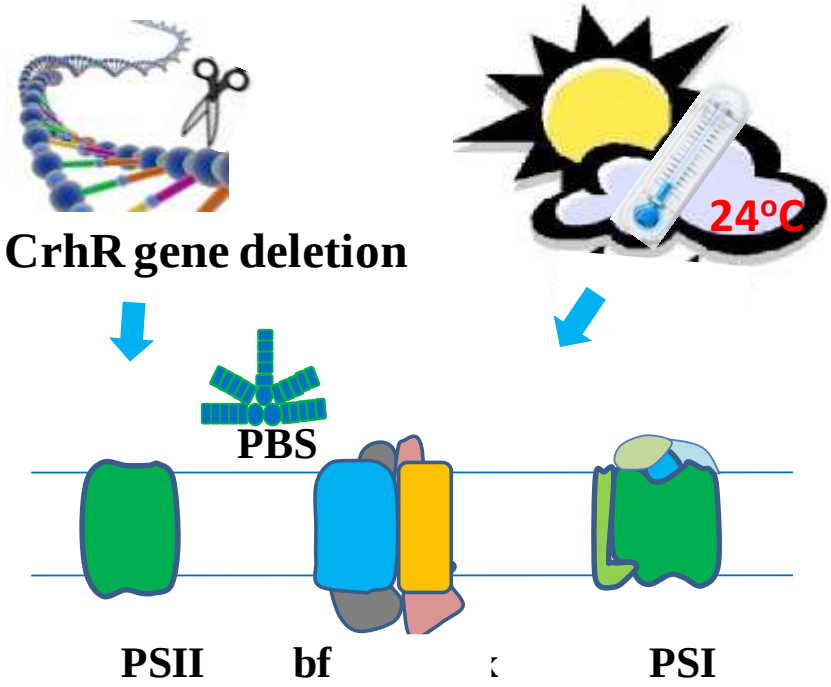
3.3. Conclusions

A systematic gene deletion approach was chosen for functional analysis of RNA helicase, CrhR in the cyanobacterium *Synechocystis* sp. PCC 6803. The gene encoding CrhR was inactivated by inserting spectinomycin resistance gene cassette in to the coding region**.** All the wild-type copies of the *crhR* gene were replaced with the spectinomycin gene cassette in the Δ*crhR* mutant. The functional consequences of deletion of *crhR* in the cell was studied by the phenotypic analysis of the Δ*crhR* under low temperature. Δ*crhR* exhibited a slow growth and bleached phenotype under low temperature indicating that RNA helicase is important for the cell to cope up the low temperature induced stress. Loss of chlorophyll was observed in the Δ*crhR* cells at low temperature. The cells of Δ*crhR* remained viable even after low temperature which is evident by the presence of intact cell membranes. Delayed cell division is prominent in Δ*crhR* cells which is evident by the presence of bilobed

nucleoids at low temperature. This is in consistent with the observed slow growth phenotype (delayed rate of cell division) of the Δ*crhR* cells. Alteration in the cell size and thylakoid membrane structure was observed due to low temperature in the wild-type cells. The thylakoid membranes were found to be less organized in the wild-type and the cells appeared to be small in size. Such modifications were not observed in Δ*crhR*. This change in the membrane architecture is further reflected by change in the photosynthetic activity in the wild-type cells. Wild-type cells exhibited a gradual increase in the PSII activity, whereas Δ*crhR* cells exhibited reduced activity at low temperature. From the above results it can be concluded that RNA helicase gene has an important function during low temperature acclimation and mutation of RNA helicase gene by deletional inactivation lead to deleterious effect on the cell

Effect of *crhR* mutation on low temperature induced changes in photosynthetic pigment protein complexes

Chapter 4



4.1. Introduction

4.1.1. Adaptation of cyanobacteria to environmental changes

One of the intriguing features of thylakoid membranes and the photosynthetic apparatus in general is the capability of adaptation during or following environmental changes. Fluctuations of various environmental factors including levels of specific nutrients, the intensity of the incident irradiation and temperature alters growth rates of phototrophic organisms. Dramatic changes in pigment content, activities of various metabolic processes and cell morphology may be observed under extreme conditions (Kehoe and Grossman, 1996; Grossman et al., 1993; He et al., 2001).

4.1.2. Light adaptation

Acclimation to light regimes is one of the most essential and complex responses of photosynthetic organisms to varying environmental conditions. Light adaptation is often accompanied by changes in content of pigments and in the composition of thylakoid proteins (Allen, 1995; Anderson et al., 1995) with following modifications in the composition of antenna complexes (Lorimier et al., 1991), redistribution of excitation energy between the photosystems (Murakami and Fujita, 1991), changes in the composition of reaction centres or in CO_2 fixation activity (Schmetterer, 1994; Murakami et al., 1997). Under light-limiting conditions cyanobacteria increase their concentration of PBPs and chlorophyll *a*. Increasing antenna sizes occurs by elongation of the phycobilisome rods and by an increase in the number of PBS per unit area of the thylakoid membrane. Under light-saturating conditions cyanobacteria reduce their antenna size and photosystem content which is accompanied with a marked decrease in the chlorophyll *a* and phycobiliprotein levels and a slight decrease in total carotenoid levels (Hihara et al., 1998; He et al., 2001; Havaux et al., 2003). Simultaneously, phycobilisome sizes and photosystem contents are reduced to avoid absorption of excess light energy.

Expression analysis in *Synechocystis* cells showed that chlorophyll *a* and PC contents decline drastically within 3 h of acclimation to high light. These changes could originate from down-regulation of the genes that encode enzymes for biosynthesis of photosynthetic pigments: chlorophyll genes and structural components of the PBS (aps and cpc genes) and from synthesis of proteins that are directly or not directly involved in the degradation of chlorophyll *a* and PBPs (Lorimier et al., 1991; Hihara et al., 2001). Expression analysis of genes encoding PBS subunits uncovered that transcription of APC genes is down-regulated by light, whereas the mRNA levels of linker proteins are not affected at high light regimes (Lorimier et al., 1991). Simultaneously, the remodeling of the PSII antenna complex is accompanied by a shortening of the PBS rods via detachment of external rod segments or whole rods from the PBS core. Various studies indicated that peptidases participate in post translational modification of the PBS antenna (Yamanaka et al., 1980). Later, it was shown that Lhc-like polypeptides (HLIP; high light inducible polypeptides) accumulate during adaptation to light stress in plants and cyanobacteria (Adamska et al., 1999; Funk and Vermaas, 1999; He et al., 2001). Cyanobacterial cells encode five genes (*hliA, hliB, hliC, hliD* and *hemH*) for polypeptides with similarity to Lhc polypeptides of vascular plants. Levels of all Hli polypeptides were elevated in high light and during nitrogen limitation (van Waasbergen et al., 2002). The transcripts of three genes, *hliA, hliB, hliC*, accumulate to high levels following exposure to sulphur deprivation and low temperature. Therefore, the products of these genes can be involved in the protection against different types of stress, including light stress. The initial accumulation of Hli polypeptides occurs during the phase of acclimation in which cells are unable to divide. The role of HliA protein in photosynthetic electron flow is unclear, although the polypeptides may be involved in the dissipation of excess absorbed light energy or function in a complex that shuttles chlorophylls to sites of degradation and/or pigment protein complex biosynthesis (He et al., 2001; Havaux et al.,

2003). Expression studies of *Synechocystis* peptidase genes under acclimation to different light regimes showed that three genes, *clpB2*, *ftsH1* and *ftsH2*, are induced within 15 min after transfer to high levels of light and become down-regulated within next 15 h (Hihara et al., 2001). As it was noted before, the products of *ftsH1* and *ftsH2* genes were found to be essential for cells since no knock-out mutants for these peptidases could be obtained. FtsH2 protein is essential for controlling the turnover of D1 protein (Bailey et al., 2002; Silva et al., 2003). In cyanobacterial cells about 110 - 140 molecules of chlorophyll *a* are attached with the reaction centre of PSI, but only 35 - 70 molecules reside in PSII reaction center. As chlorophyll *a* is mainly associated with PSI, the acclimation to high light is accompanied by a decrease in PSI relative to PSII (Fujita et al., 1994; Murakami et al., 1997a; McConnell et al., 2002). Such adaptation serves to regulate the distribution of excitation energy between photosystems and to correct any energy imbalances (Hihara et al., 1998). Changes in photosystem contents are regulated by decreasing mRNA levels of genes encoding subunits of photosystems (Hihara et al., 1998; Hihara et al., 2001; Muramatsu and Hihara, 2003) and simultaneously, by activation of proteolytic components involved in the degradation of photosynthetic proteins during light stress (Lindahl et al., 2000; Bailey et al., 2001; Lensch et al., 2001; Kanervo et al., 2003; Silva et al., 2003). Recently, it has been shown that transcript levels of photosystem I genes rapidly decrease to less than 10% of initial levels within 1 h after a shift to HL, whereas the response of PSII transcript levels was not coordinated (Hihara et al., 1998; Muramatsu and Hihara, 2003). Fast changes in the transcription of PSI genes, but not PSII genes, seem to be important for *Synechocystis* cells to regulate their photosystem contents in response to high light acclimation. Probably, the content of PSII can be promptly reduced under HL conditions due to an accelerated turnover rate of its reaction centre subunits (Mohamed and Jansson, 1989; Komenda et al., 2000). The turnover process of PSII includes the degradation of damaged D1 polypeptide, de novo synthesis of D1 and assembly

of the heterodimeric complex with other PSII polypeptides (Melis, 1991; Komenda et al., 2000). There are various proteases and chaperones that are involved in D1 turnover *in vivo*. Two families of proteases are being studied: the FtsH family of Zn^{2+}-activated nucleotide-dependent proteases; and the HtrA (or DegP) family of serine-type proteases. Recent evidence showed that the HtrA family of proteases is involved in the resistance of *Synechocystis* to light stress and play a part, either directly or indirectly, in the repair of PSII *in vivo* (Bailey et al., 2001). Chloroplast FtsH showed light-inducible gene expression (Lindahl et al., 2000). It was proposed that this peptidase is involved in the degradation of unassembled thylakoid proteins (Ostersetzer and Adam, 1997) and perhaps, in the second step of the degradation of PSII reaction center core protein D1 (Lindahl et al., 2000; Silva et al., 2003).

4.1.3. Heat stress

Response to elevated temperature is an environmental factor that is also studied in cyanobacteria. Exposure of cyanobacterial cells to temperatures exceeding 60 – 65^{o}C even for 10 min results in a bleached phenotype. Such alternation is the result of a breakdown of components of PBS (Zhao and Brand, 1989; Nishiyama et al., 1993). Higher temperatures bleach bilin-containing pigments fast, but also resulted in a gradual bleaching of chlorophyll and carotenoids. PSII has been shown to be the most sensitive thylakoid assembly to heat among the photosynthetic activities (Berry and Bjorkman, 1980). The dissociation of two of the four Mn atoms from the PSII complex by heat results in complete inactivation of oxygen evolution without significant loss of proteins (Nash et al., 1985). Therefore, the mechanism of photosynthetic adaptation to high temperature is related to ability to protect the PSII oxygen evolving complex against heat-induced inactivation.

Different factors, which are regulated by growth temperature, may contribute to observed adaptation. Early investigations suggested that high temperature increases the level

of saturated fatty acids in membrane lipids and enhances the thermal stability of photosynthesis (Shneyour et al., 1973). Later, it has been shown that thermal stability is not only affected by changes in the saturation level of membrane lipids, but other factors are also responsible for adaptation of photosynthesis to high temperature. There are a number of heat stress proteins, chaperones and peptidases that are involved in the refolding or degradation of polypeptides misfolded by heat. One of the best studied peptidases functioning during heat stress is the bacterial HtrA peptidase (Strauch et al., 1989; Lipinska et al., 1990; Spiess et al., 1999).

4.1.4. Low temperature

Research on temperature stress in cyanobacteria has mainly focused on heat stress while less attention has been paid to low temperature. Nevertheless, some mechanisms of cold responses and acclimation have been identified (Phadtare, 2004). At low temperatures chaperones act to protect proteins that are susceptible to damage. Lower temperatures make cell membranes more rigid, and as a consequence the *desA* and *desB* genes encoding desaturase-enzymes in *Synechocystis* are activated (Sakamoto and Bryant, 1997). Desaturases increase the number of double bonds in fatty acid hydrocarbon chains thus increasing membrane fluidity. While its role in cellular sensing encompasses a variety of stresses, the membrane-bound Hik33 histidine kinase also perceives the degree of membrane fluidity and regulates the expression of the desaturases (Suzuki et al., 2000; Suzuki et al., 2001; Kanesaki et al., 2007).

The main aim of this objective is to study the low temperature induced changes in the photosynthetic machinery and also study the impact of mutation of *crhR* on the cell during low temperature.

4.2. Results and Discussion

4.2.1. Effect of low temperature on *Synechocystis* wild-type and Δ*crhR*

Acclimation responses to stress conditions include changes in the cell morphology, metabolism and pigment content. Changes in the pigment protein complexes can be analysed by absorption spectra of whole cells. The absorption spectra of the wild-type and Δ*crhR* cells grown under optimal and low temperature stress were taken in order to compare the pigment content alterations. Absorption spectra of wild-type cells and Δ*crhR* cells were recorded to further examine the changes in pigment protein complexes at low temperature incubation. Both strains exhibited absorption peaks corresponding to chlorophyll *a* at 685 nm and 446 nm, as well as an absorption peak of PC at 625 nm. An increase in the absorption maximum of both chlorophyll *a* and PC peaks were observed in the WT-24^{o}C-cells when compared to the WT-34^{o}C-cells (Fig.4.1). Absorption spectra of Δ*crhR*-24^{o}C-cells exhibited a decrease in the chlorophyll *a* absorption maximum when compared to the Δ*crhR*-34^{o}C-cells. This further supports the observation that in Δ*crhR*-24^{o}C cells the cellular concentration of chlorophyll binding proteins is significantly lower when compared to Δ*crhR*-34^{o}C cells (Fig.4.1).

4.2.2. Blue shift of Chlorophyll absorption peak in wild-type cells at low temperature

Absorption spectra were recorded for wild-type and Δ*crhR* cells that were grown under optimum growth conditions (34^{o}C) and then shifted to low temperature conditions (24^{o}C). Spectra were recorded at 3,6,23,29,47,53 and 72 h after shift from 34^{o}C to 24^{o}C. This lead to the identification of low temperature induced alteration in the pigment protein complexes during the onset of incubation at 24^{o}C. After 72 h of low temperature treatment, we observed a shift in the chlorophyll absorption peak from 685 to 680 nm in the wild-type cells (Fig.4.2A). However this blue shift was not observed in the absorption spectra of Δ*crhR* cells during the course of low temperature incubation (Fig.4.2B). Previous reports indicated that the expression of IsiA (Iron stress inducible protein A) lead to the peak shift under iron stress

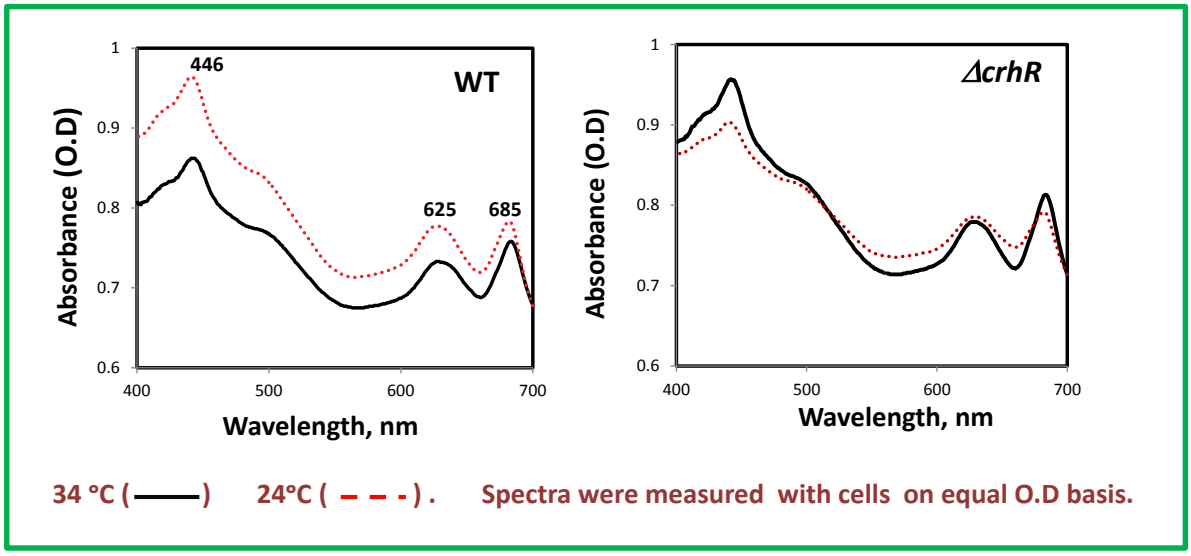


Fig.4.1. Changes in absorption spectra of wild-type and Δ*crhR* cells at 34^{o}C and during incubation at 24^{o}C. (A) Absorption spectra of wild-type cells grown at 34^{o}C for 16 h and the wild type cells that had been grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h (B) Δ*crhR* mutant cells grown at 34^{o}C for 16 h and the Δ*crhR* cells that had been grown at 34^{o}C and then incubated at 24^{o}C for 72 h. The spectra were corrected for light scattering at 700 nm.

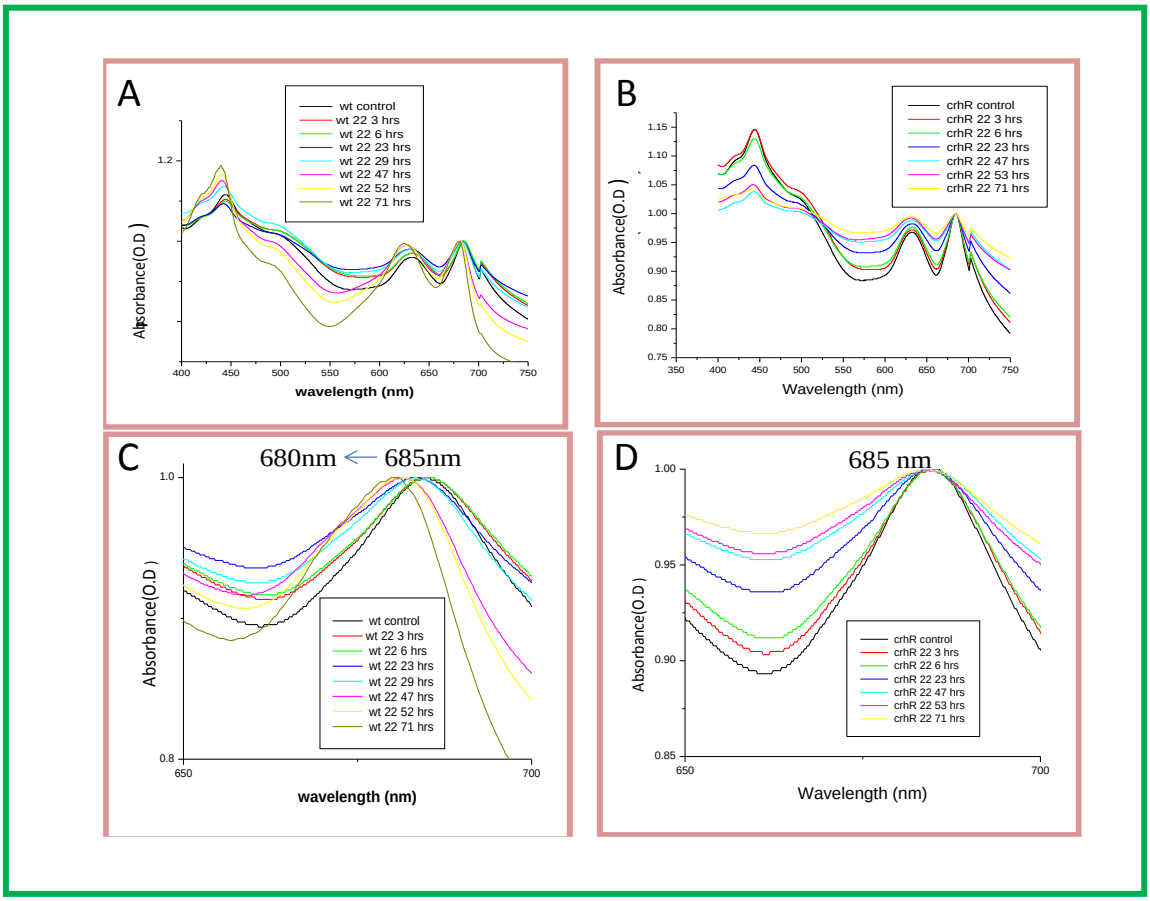


Fig.4.2. Changes in chlorophyll absorption spectra to monitor blue shift in wild-type during low temperature (A) Absorption spectra of wild-type cells grown at 34^{o}C for 16 h and the wild type cells that had been grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h: (B) Δ*crhR* mutant cells grown at 34^{o}C for 16 h and the Δ*crhR* cells that had been grown at 34^{o}C and then incubated at 24^{o}C for 72 h. (C) and (D) Chlorophyll Absorption peak of wild-type and Δ*crhR* at 685 nm.

conditions. It has also been reported that the monomerisation of PSI contributes to the peak shift under iron stress (Sandstrom et al., 2002). The blue shift could be due to changes in the pigment- protein interactions that might have under gone various modifications like loss of longer wavelength chlorophylls to perform optimal photosynthesis upon prolonged exposure to low temperature. However in Δ*crhR* blueshift was not observed when the culture was shifted to low temperature.

4.2.3. Loss of chlorophyll is prominent in Δ*crhR* cells at low temperature

In order to monitor changes in pigment protein complexes in more detail, further examined the effect of low temperature on pigment content of the Δ*crhR* cells in comparison with wild-type cells. From the results presented in Fig.4.3A, a significant increase in the chlorophyll content was observed during incubation of wild-type cells at 24^{o}C, suggesting incubation of wild-type cells at low temperature enhances the levels chlorophyll containing protein complexes. Almost similar chlorophyll content was observed between wild-type and Δ*crhR* cells at 34^{o}C. However, during incubation of cells at 24^{o}C, Δ*crhR* cells contained relatively lower chlorophyll content, as compared to the wild-type cells suggesting that loss of chlorophyll protein complexes in the Δ*crhR* cells at low temperature (Fig.4.3A).

Results presented in Fig.4.3B shows PC content in wild-type and Δ*crhR* during low temperature incubation. A significant increase in the PC content during incubation of wild-type cells at 24^{o}C, suggests an increase in phycobilisome content during incubation of cells at 24^{o}C. A slight but significant increase in the PC content was observed Δ*crhR* cells at low temperature (Fig.4.3B). Fig.4.3B indicates that there is fourfold increase in the PC content (at 72 h time point) of the wild-type cells at 72 h of 24^{o}C incubation. However Δ*crhR* cells displayed only two fold increase in the PC content. The observed loss in the PC content is mainly from the reduced cell density of the Δ*crhR* cells.

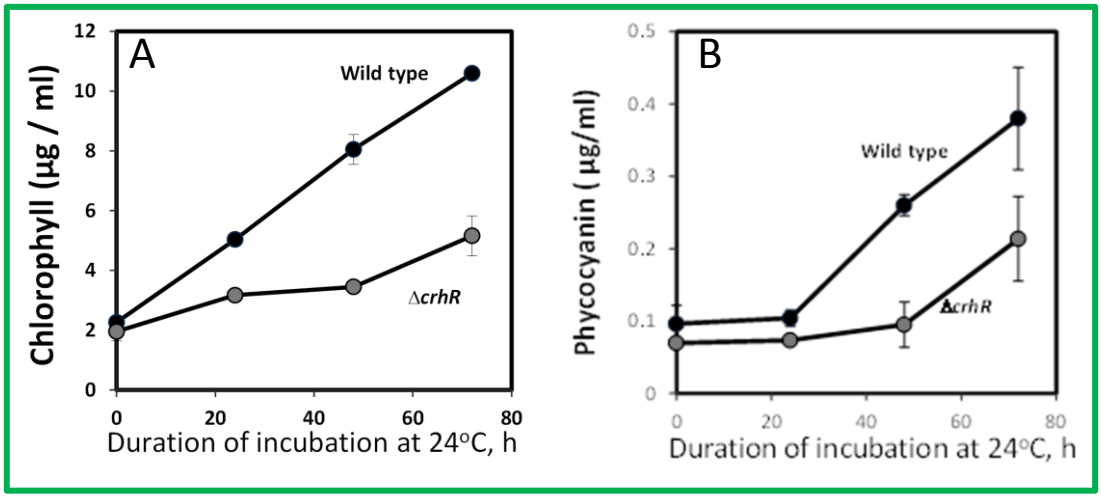


Fig.4.3. Pigment analysis at 34°C and during low temperature treatment in wild- type (black circles) and Δ*crhR* (grey circles). (A) Chlorophyll content ;(B) PC content.

These results indicate that there is a predominant loss of chlorophyll rather than the PC in the Δ*crhR* cells under low temperature.

4.2.4. Effect of low temperature on oligomerization of photosystems

The extent of changes in photosystems in *Synechocystis* wild-type and Δ*crhR* cells under low temperature were monitored by sucrose density gradient separation of solubilized thylakoid complexes. Fractionation of solubilized thylakoid membranes on sucrose density gradient resulted in three pigment protein containing fractions (Fig.4.4). According to previous reports the orange coloured fraction on the top of sucrose density gradient (F1) comprises of free pigments, upper green fraction (F2) enriched with PSII and PSI monomer mixture and lowest green fraction (F3) contains PSI trimer (Fuhrmann et al., 2009). The level of F2 containing PSII and PSI monomer was increased in WT-24^{o}C-cells (Fig.4.4). Fractionation of thylakoid membranes isolated from Δ*crhR*-24^{o}C-cells showed a significant decrease in the PSI trimer and an increase in the PSII/PSI monomer fraction as compared to the Δ*crhR*-34^{o}C-cells (Fig.4.4). However, the relative increase in the PSII/PSI monomer fraction and decrease in the PSI trimer fraction does not reflect the concentrations within the living cells because, the sucrose density gradients were loaded on equal chlorophyll basis, even though there was significantly low chlorophyll in the Δ*crhR*-24^{o}C-cells (see Fig.3.9 bleached phenotype). Thus, the loss in PSI trimers may be much more in reality than observed and the increase in PSII/PSI monomers may not be occurring *in vivo*.

Since the Sucrose density gradient centrifugation was carried out on equal chlorophyll basis and the relative amount of PSII and PSI of F2 seemed to be increased in the Δ*crhR* when compared to wild-type, to rule out this sucrose density gradient centrifugation of the solubilised membranes were carried out on equal protein basis (Fig.4.5). The F2 comprising of PSI monomer and PSII dimer were not affected under low temperature in both

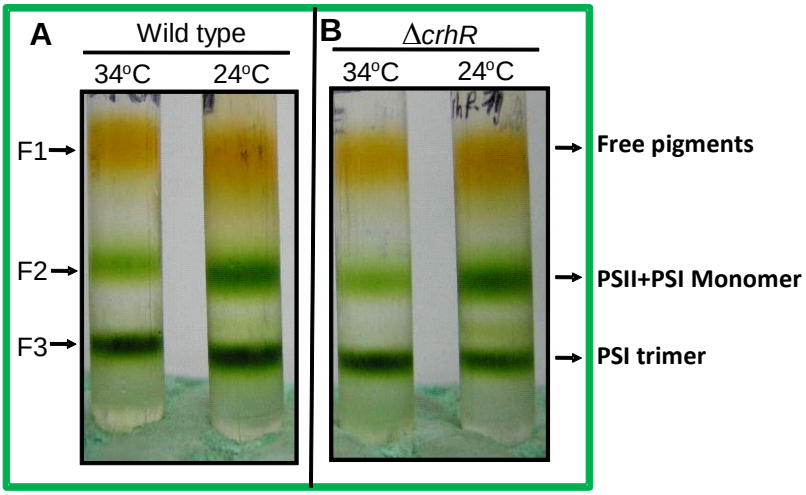


Fig.4.4. Fractionation of the thylakoid protein complexes by sucrose density ultracentrifugation. (A) Thylakoid protein complexes of wild-type cells grown at 34°C for 16 h and wild type cells that had been grown at 34°C for 16 h and then incubated at 24°C for 72 h. (B). Thylakoid protein complexes of Δ*crhR* cells grown at 34°C for 16 h and Δ*crhR* cells that had been grown at 34°C for 16 h and then incubated at 24°C for 72 h. The thylakoid protein complexes were then separated by ultracentrifugation at 160,000 x *g* for16 h at 4°C on a step gradient of sucrose density. Samples were loaded on equal chlorophyll basis.

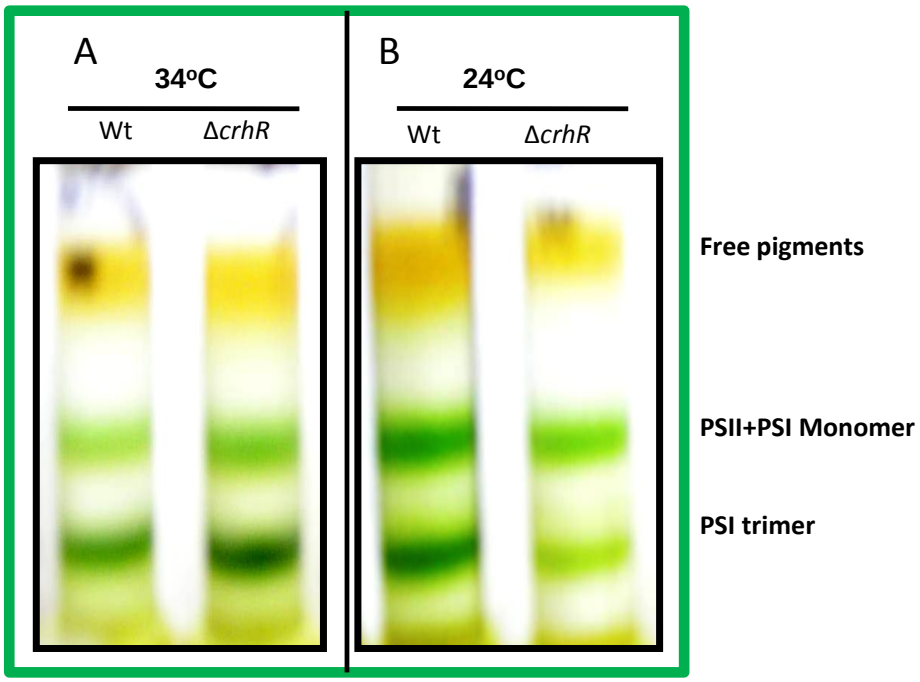


Fig.4.5. Fractionation of the thylakoid protein complexes by sucrose density gradient ultracentrifugation (A) Thylakoid protein complexes of wild-type cells grown at 34^{o}C for 16 h and wild type cells that had been grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h. (B) Thylakoid protein complexes of Δ*crhR* cells grown at 34^{o}C for 16 h and Δ*crhR* cells that had been grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h. The thylakoid protein complexes were then separated by ultracentrifugation at 160,000 x *g* for 16 h at 4^{o}C on a step gradient of sucrose density. Samples were loaded on equal protein basis.

wild-type and Δ*crhR* formation of trimeric form of PSI was found to be hugely effected in the mutant.

The changes observed in Sucrose density ultracentrifugation were further confirmed by Green Gel electrophoresis of the solubilised thylakoid membranes after resolving on to 10% LiDS gel. Green gel electrophoresis showed four distint bands on the slab gel (Fig.4.6). From top to bottom of the gel the pigment band corresponds to the PSI trimer, PSI monomer, PSII and free pigment. In consistent with sucrose density gradient separation of the solubilized membranes there was no significant difference in the trimer between wild-type 34 ^{o}C cells and wild-type 24^{o}C cells. A significant decrease in the PSI trimer content was observed in *crhR*-24^{o}C cells when compared to *crhR* 34^{o}C cells, accompanied by a significant increase in the PSI monomer content. This data suggests that mutation in RNA helicase crhR leads to monomerisation of PSI trimer at low temperature and drastic decrease in the overall content of PSI. A slight but significant decrease in PSI trimer content was observed in the wild-type cells-24^{o}C when compared to wild-type-34^{o}C cells.

4.2.5. Enhanced PSII dimer content in wild-type cells was observed at low temperature

In sucrose density centrifugation changes in PSII were not resolved hence the pigment protein complexes were further separated by BN-PAGE. To further confirm the oligomeric states of the photosystems, BN PAGE was carried out for the solubilised thylakoids (Fig.4.7). The pattern of PSI trimeric and monomeric complexes on the gel was identical in both the wild-type and Δ*crhR* cells maintained under optimal conditions exhibiting the PSI trimers as predominant species (Fig.4.7). PSII dimer content was increased in wild-type 24 ^{o}C when compared to wild-type 34^{o}C cells. In contrast, no significant change in the PSII dimer content was observed on incubation of Δ*crhR* cells at 24^{o}C for 72 h. As observed by Sucrose density gradient separation of PSI complexes and by green gel electrophoresis, PSI trimer content was significantly reduced in Δ*crhR*-24 ^{o}C when compared to Δ*crhR*-34^{o}C cells. Thus

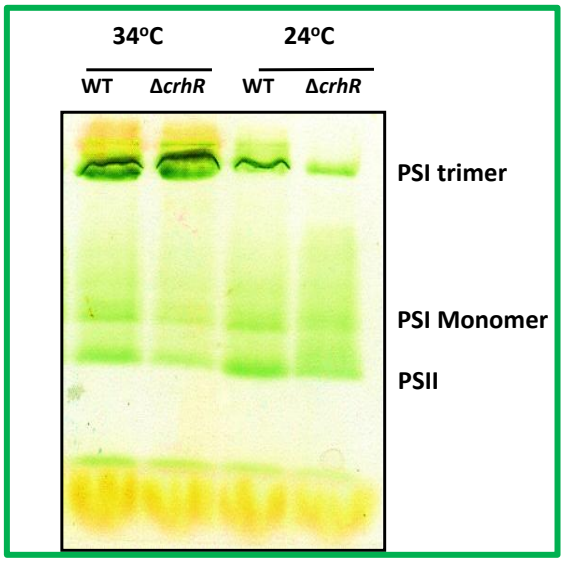


Fig.4.6. Mild denaturing green gel electrophoresis analysis of chlorophyll pigment complexes in thylakoid membranes. Native PAGE (10%) of thylakoids isolated from WT-34^{o}C cells, WT- 24^{o} C cells, Δ*crhR*-34^{o}C cells and, Δ*crhR*-24^{o}C cells. Sample preparation was done by solubilising thylakoid membrane by mixing equal volume of 2X sample buffer and 10% w/v DDM at 4^{o}C under constant rotation for 25 min. Unsolubilized membranes were pelleted out by ultracentrifugation at 45,000 x g for 30 min at 4^{o}C. Supernatant containing solubilised thylakoid membranes equivalent of chlrophyll was loaded on each lane of the 8% SDS-PAGE gel (4% stacking gel, 10% resolving gel).

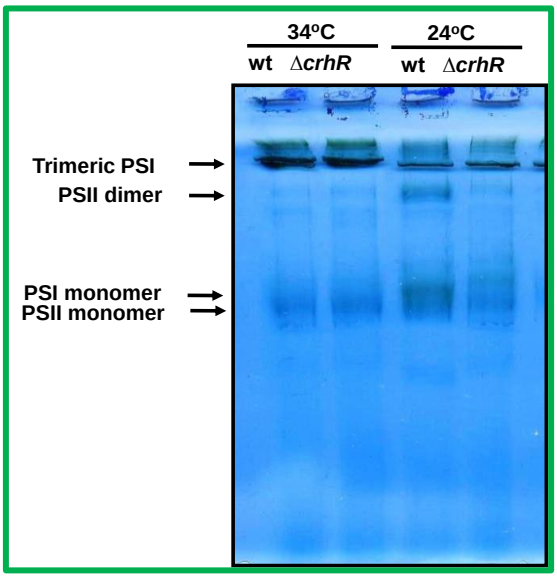


Fig.4.7. Pigment protein complexes of wild-type and Δ*crhR* at 34^{o}C and 24^{o}C separated by 8% BN- PAGE. To the solubilised thylakoid complexes added 1/10 volume of 5% serva blue G (100 mM Bis Tris-HCl, pH 7.0, 0.5 M 6-amino-n-caproic acid, 30% glycerol) and loaded on equal chlorophyll basis.

it is clear that PSI trimer content was significantly reduced in Δ*crhR* at low temperature. PSII dimers formed in the wild-type at low temperature could be an acclimative response to low temperature stress. Such an acclimative oligomerization of PSI was not detected in Δ*crhR* mutant at 24 ^{o}C.

4.2.6. PBS uncoupling at low temperature in wild-type cells and effect of Δ*crhR* mutation

Since, PBS are the major light-harvesting complexes involved in cell acclimation, the degradation of the PBS can reflect one of the protection mechanisms for controlling photosynthetic activity during stress conditions. The reduction of the PBS can lead to a decrease of absorbed energy, thus preventing cells from photo damage. The adaptation of the PBS to stress occurs through a decrease of the rod size, the number of rods and the number of PBS that are controlled at the transcriptional and post-translational levels. The PBS are mobile structures on the surface of the thylakoid membrane. The association of PBS with PS II and PSI is transient and PBS can be found in a membrane-bound and non-associated form with PSI/PSII. PBS efficiently harvest light energy and transfer it to the chlorophyll *a* of PSII or PSI when they are attached to these complexes. Thereby the membrane-bound and free PBS (soluble in the cytoplasm) may provide information on energy absorption and transfer through PBS to the reaction centres of the two photosystems.

Fig.4.8 shows the room temperature fluorescence emission spectra at of wild-type and Δ*crhR* cells, when excited at 580 nm (Fig.4.8A, B) or 436 nm (Fig.4.8C, D). As shown in Fig.4.8A, there was a significant rise in PBS emission peak at ~660 nm, in WT-24^{o}C-cells when compared to wild-type-34^{o}C-cells. However, there was no difference in the fluorescence emission at 660 nm in Δ*crhR* -34^{o}C-cells and Δ*crhR* -24^{o}C-cells when excited cells excited at 580 nm (Fig.4.8B).

In chlorphyll emission spectra, significant increase in the emission spectral properties at 605, 645, and 680 nm which corresponds to PC, APC and terminal emitters of PBS,

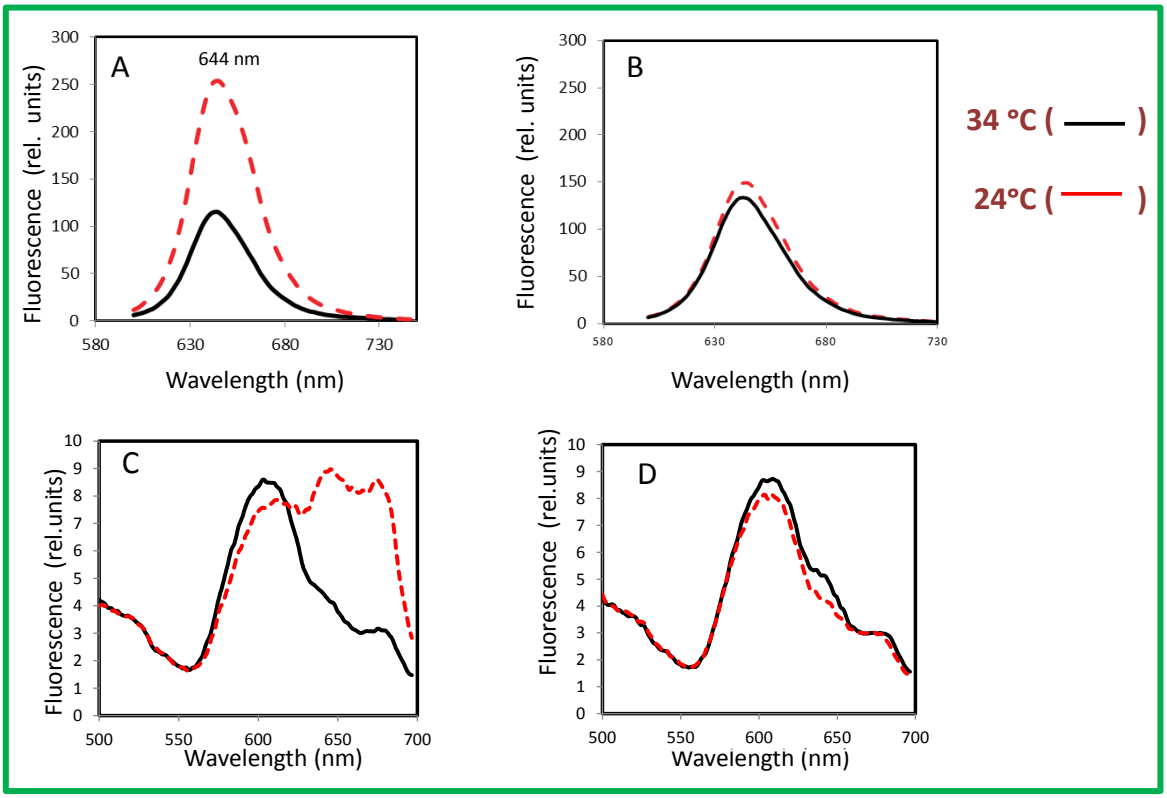


Fig.4.8. Changes in fluorescence emission spectrum of wild-type and Δ*crhR* cells at 34°C and 24°C. Fluorescence emission spectra of wild-type (A) and Δ*crhR* cells (B) excited at 580 nm. Fluorescence emission spectra of wild-type (C) and Δ*crhR* mutant cells (D) excited at 436nm. Spectrum of cells grown at 34°C (solid line) and cells incubated at 24°C for 72 h (dashed line).

respectively was monitored in wild-type-24^{o}C-cells when compared to wild-type-34^{o}C-cells (Fig.4.8C). There was no difference in Δ*crhR*-34^{o}C-cells and Δ*crhR*-24^{o}C-cells (Fig.4.8D). Taken together absorption and fluorescence emission spectra it is clear that the increase in fluorescence emissions at 605, 645 and 680 nm were may be due to uncoupling of PBS from PSII in wild-type cells at low temperature. Such uncoupling of PBS was not seen in the Δ*crhR* mutant.

4.3. Conclusion

Different environmental parameters can modify the activities of photosynthetic complexes and the levels of pigments and proteins associated with these complexes (Grossman et al., 1993). The photosynthetic apparatus is highly dynamic and alteration of light harvesting complex synthesis and degradation occurs in response to environmental stimuli including changes in light quality (Tandeau de Marsac, 1993), light intensity (Horton et al., 1996), and nutrient availability (Allen and Smith, 1969). Such an alteration helps to efficiently balance the absorption of excitation energy.

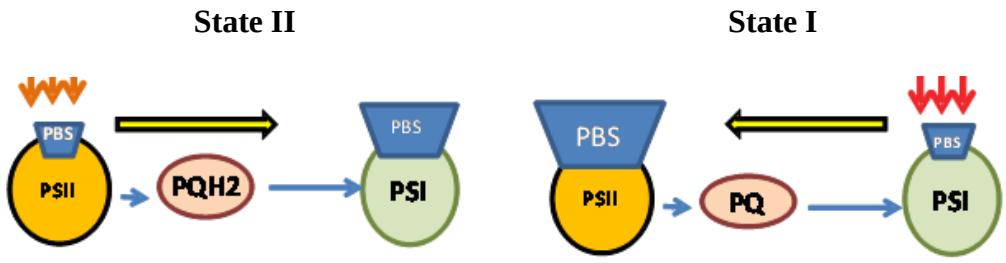
Here in the present study with wild-type and Δ*crhR* cells grown at low temperature conditions revealed several acclimatory mechanisms. Changes in pigment protein complexes indicated a preferential loss of chlorophyll over PC in Δ*crhR* upon low temperature treatment. An alteration in PC/Chl ratio was also observed in the wild-type and Δ*crhR* indicating that the association of PBS to chlorophyll has altered. However in Δ*crhR* the ratio of PBS to chlorophyll has not altered but overall photosystems were altered. Loss of longer wavelength chlorophylls was evidenced by a blue shift in the red region of absorption spectrum was observed in the wild-type cells upon low temperature treatment. However such shift was not seen in Δ*crhR*-24^{o}C-cells. Uncoupling of PBS from PSII in the wild-type cells was observed upon low temperature. Such uncoupling was not seen in Δ*crhR*. An increase in the PSI monomer content and PSII dimer content upon low temperature was observed in the wild-

type indicating changes in the oligomerisation of the photosystems. However increase in PSII dimer content is not seen in the mutant while the loss of PSI trimer is more significant.

All the above results indicate that the wild-type cells adopt different mechanisms to cope up the low temperature stress while Δ*crhR* was unable to exhibit these adaptive responses. Since ability of cyanobacteria to sense and respond to changes in their environment through a complex series of regulatory pathways is initiated by changes in electron flow and RNA helicase was reported to be redox regulated, we assumed that redox regulation of *crhR* expression allows *Synechocystis* to respond rapidly to environmental changes, on the order of minutes, similar to the PQ redox state-mediated response observed for the photosynthetic genes, *psaAB* and *psbA*, in mustard chloroplasts (Pfannschmidt et al., 1999). Hence we further aimed to study the redox regulated changes in the wild-type and Δ*crhR* upon shift to low temperature in the next objective.

Effect of *crhR* mutation on low temperature induced functional balancing between photosystems

Chapter 5



5.1. Introduction

The effective absorption of sunlight by antenna pigments is the critical first step in photosynthesis. All oxygenic photosynthetic organisms share a common core antenna pigment complex of about 40 chlorophyll *a* in PSII and about 100 chlorophyll *a* in PSI (Rogner et al., 1990). Photosynthetic organisms do not, however, limit their photon capturing ability to this level, but rather use some form of additional peripheral antenna pigments to increase the effective "absorption cross section" of one or both photosystems. Higher plants and algae have evolved diverse mechanisms to increase their ability to absorb sunlight. Both cyanobacteria and higher plants can regulate the efficiency of excitation energy transfer to the two photosystems. The light state transition appears designed to adjust the relative activities of PSII and PSI in response to a dynamic environment or to changing metabolic demands (Yu et al., 1993). The mechanism in higher plants involves a reversible association of LHCII with PSII and PSI triggered by the redox state of intersystem electron transport carriers and driven by the reversible phosphorylation of LHCII (Allen, 1992; Wollman, 2001). There is no consensus for the mechanism of the state transition in PBS-containing cyanobacteria (Van Thor et al., 1998; Mullineaux, 1999).

5.1.1. Distribution of absorbed light energy between photosystems: State transitions

The phenomenon of light-induced fluorescence changes that later termed "state transitions" was discovered independently by Murata (1969) in the phycobilisome containing red alga *Porphyridium cruentum*, and by Bonaventura and Myers (1969) in the green alga *Chlorella pyrenoidosa*. State 1/state 2 transitions reflect a rapid physiological adaptation mechanism that regulates the way in which absorbed light energy is distributed between PSI and PSII. State transitions occur in both green plants and cyanobacteria, although the light-harvesting complexes involved are very different.

5.1.2. State transitions in chloroplasts of higher plants

The state transitions in the chloroplasts of higher plants regulate the energy from the light-harvesting antenna to the PSI and PSII. This short-term regulation is based on the reversible phosphorylation of LHCII by a thylakoid-bound kinase (Allen et al., 1981; Pursiheimo et al., 1998; Carlberg, 1999; Bellafiore et al., 2005). An important feature of the photosynthetic membranes in higher plants is the lateral heterogeneity of the two photosystems: PSII complexes are localised mostly in grana stacks, while PSI complexes were found in non-stacked stromal thylakoids. When the PQ pool is oxidized, LHCII is associated with PSII and this is known as state 1 (Fig.5.1). When a change in light intensity or quality leads to reduction of the PQ pool, the LHCII kinase is activated, and LHCII becomes phosphorylated and the mobile pool of LHCII (Lhcb1 and Lhcb2) moves to PSI (state 2) (Vener et al., 1997; Allen and Forsberg, 2001; Haldrup et al., 2001; Wollman, 2001; Khrouchtchova et al., 2005) (Fig.5.1). The PQ becomes oxidized and the LHCII kinase is inactivated. The LHCII phosphatase which is continuously active, catalyses the dephosphorylation of LHCII, thereby returning excitation energy to PSII (Silverstein et al., 1993). The correlation between PQ redox level, state transitions and phosphorylation of LHCII has been demonstrated in numerous studies. Nevertheless, a demonstration that a reduced plastoquinine pool or highly LHCII phosphorylation causes or is required for state transitions has not been presented and the exact function of state transitions is still not clear (Haldrup et al., 2001; Scheller et al., 2001). Although the phosphorylation of LHCII could be involved in regulation of state transitions, it could also be a stabilizing factor that prevents the degradation of LHCII that later becomes exposed in the stroma lamellae (Haldrup et al., 2001). It was proposed that state transitions more likely could be involved in optimizing the photosynthetic yield and thus, growth under low light conditions. Bellafiore et al., (2005) proposed that state

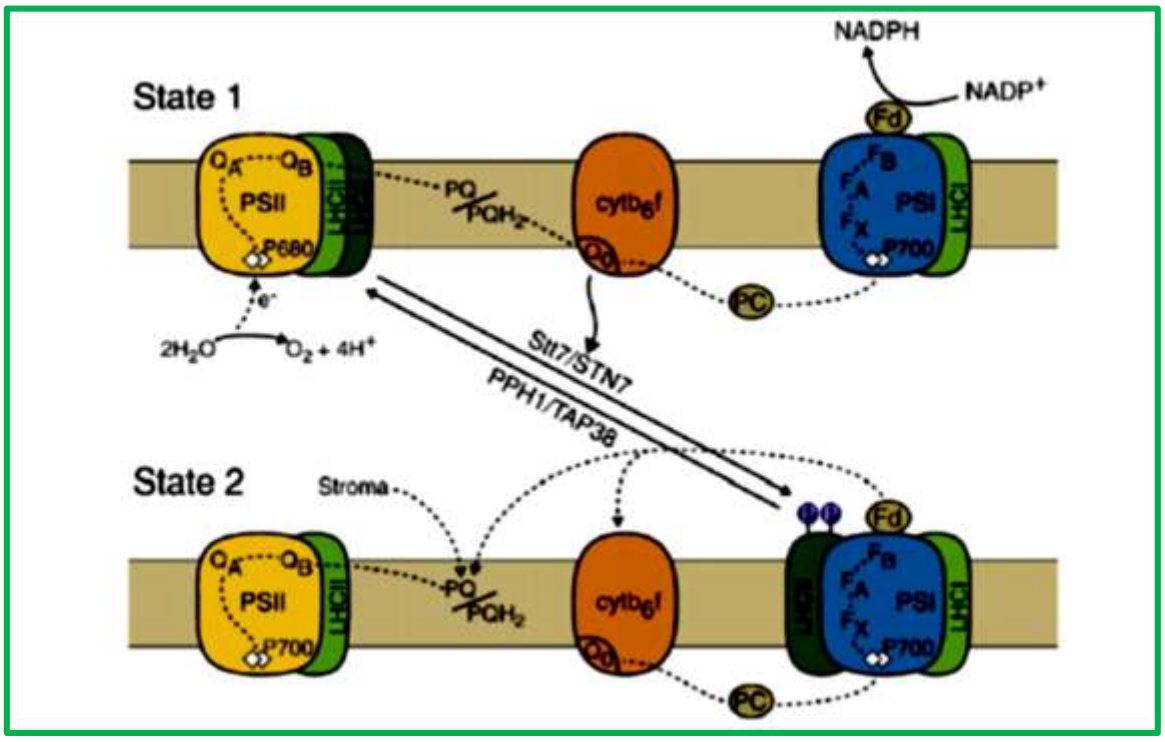


Fig.5.1. The scheme of state transitions in *C. reinhardtii.* The linear and cyclic electron transfers are indicated by arrows. Transition from state 1 to state 2 occurs when the redox state of the PQ pool is reduced, for example, as a result of preferential excitation of PSII relative to PSI. Docking of plastoquinol (PQH_2) to the Qo site of Cyt b_6f leads to the activation of the protein kinase Stt7/STN7, which phosphorylates LHCII either directly or indirectly. Phosphorylated LHCII dissociates from PSII and binds to PSI. State 1 to state 2 transition leads to a switch from linear electron flow to cyclic electron flow. Upon preferential excitation of PSI relative to PSII, the kinase is inactivated and the PPHI/TAP38 phosphatase dephosphorylates LHCII, which moves back to PSII. Adapted from Rochaix, 2011.

transitions are important for adaptation of plants to natural environments where light quality and quantity change frequently.

5.1.3. State transitions in cyanobacterial cells

Cyanobacteria, similar to higher plants, also use state 1/state 2 transitions as the major mechanism for energy distribution between PSII and PSI. In cyanobacteria, the process of state transition was discovered by Fork and Satoh (1983). State transition in cyanobacteria is a physiological adaptation mechanism that changes the interaction of the PBS with the PSI and PSII core complexes. The energy redistribution mechanism involved is not as well characterized as those in higher plants. Different from higher plants, in cyanobacteria the main light-harvesting antennas are extrinsically associated with thylakoid membranes and represent supramolecular structures known as PBS (Grossman et al., 1993). PBS are primarily composed of bilin-containing PBP). The PBS have been known to act preferentially as a light-harvesting antenna for PSII, although some of the energy trapped by the PBS is also transferred to PSI (Glazer, 1989, Rakhimberdieva et al., 2001). The PBS-covered thylakoids of cyanobacteria are not stacked, and thus some difference from higher plants may occur in the mechanism for state transitions in this organism. The mechanisms of state transitions in PBS and chlorophyll *a/b*-containing organisms are presently controversery and several models have been proposed (Fig.5.2). The PBS antenna are able to uncouple from and couple to PSII and PSI, respectively, during state transition (the "mobile antenna model") (Allen et al., 1985; Mullineaux et al., 1997), or state transitions may occur due to a spill-over of excitation energy from PSII to PSI (the,"spill-over model") (McConnell et al., 2002; Tsimilli et al., 2009). Accordingly to the third model (the "detachment PBS model") (Mullineaux et al., 1997), some PBS become detached from PSII without association with PSI during illumination with PBS-absorbed light (shift to state 2). A mutant of *Synechocystis* sp. PCC 6803 with no detectable PSII chlorophyll-binding proteins showed quite efficient

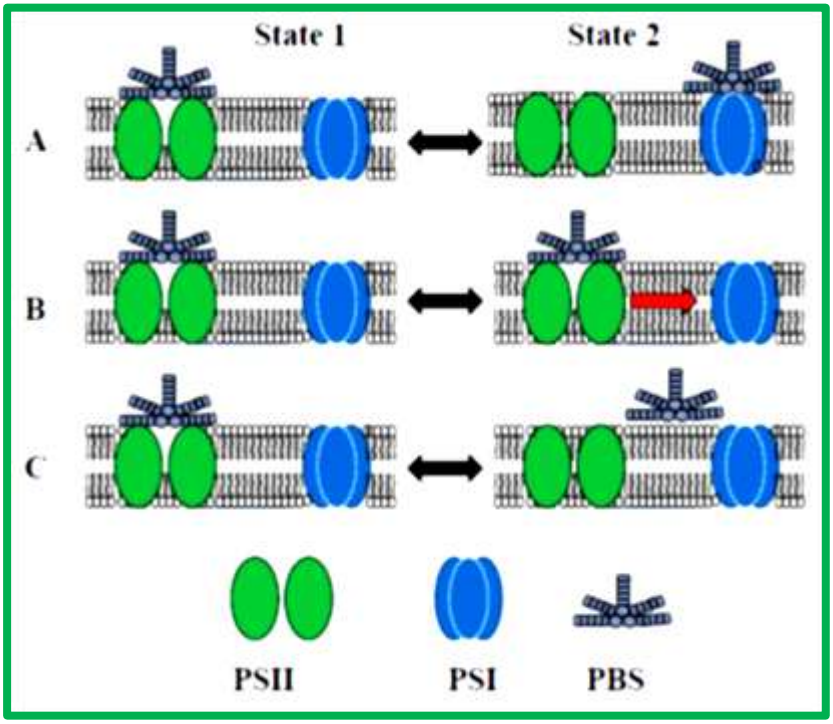


Fig.5.2. Proposed model for the mechanisms of state transitions in cyanobacteria. (A).mobile antenna model: The PBS antenna are able to uncouple from and couple to PSII and PSI, respectively. (B). spill-over model: State transitions may occur due to a spill-over of excitation energy from PSII to PSI. (C). detachment PBS model: some PBS become detached from PSII without association with PSI during illumination with PBS-absorbed light.

energy transfer from PBS to PSI (Mullineaux, 1994). It was found that in cyanobacteria PSII is immobile, but PBS diffuses rapidly on the membrane surface. The association of PBS with reaction centres is highly dynamic; there are no stable phycobilisome-reaction centre complexes in cyanobacteria (Sarcina et al., 2001; Joshua and Mullineaux, 2004). It has been shown that PBS mobility is necessary for state transitions in cyanobacteria (Joshua and Mullineaux, 2004; Mullineaux and Emlyn-Jones, 2005). The physiological role of state transitions is still a matter of debate. State transitions are a way to maximize the efficiency of light-harvesting at low light intensities (Mullineaux and Emlyn-Jones, 2005). Recently, the gene required for the state transitions has been identified in the cyanobacterium *Synechocystis* sp. PCC 6803. This gene (*sll1926*) encodes a putative integral membrane protein of 16 kDa, which was designated as RpaC (regulator of P association C) (Emlyn-Jones et al., 1999; Mullineaux and Emlyn-Jones, 2005). The mechanism of state transition can easily be monitored by using chlorophyll *a* fluorescence induction curves.

5.1.4. State transitions can be studied by fast and slow chlorophyll *a* fluorescence analysis

Chlorophyll *a* fluorescence is a highly versatile tool, not only for researchers studying photosynthesis, but also for those working in broader fields related to physiology of plants and green algae. Chlorophyll fluorescence analysis is sensitive, real-time, non-invasive, and relatively simple, but indirect. Fluorescence analysis depends on the phenomenon that when a pigment absorbs the energy of a photon and enters an excited electronic state, there are essentially four routes for the return to ground state: (i) photochemical reactions in which the excited electron leaves the pigment molecule and enters an electron transport chain, as occur in specific chlorophylls in photosynthetic reaction centers; (ii) heat dissipation, in which the excited electron returns to ground state by releasing heat; (iii) transfer of the excitation energy to an adjacent pigment, as occurs in the light-harvesting antenna systems of

photosynthetic organisms; and (iv) emission of a fluorescence photon, of a wavelength longer than that of the photon initially absorbed. These four processes are in competition, and for a given excited molecule, the path with the largest first-order rate constant predominates (Fig.5.3). For biological systems, the overall chlorophyll fluorescence yield is usually low, and *in vivo* chlorophyll fluorescence from PSII (PSII) predominates (Evans and Brown, 1994; Jones and Myers, 1963; Papageorgiou, 1996). In cyanobacteria, PBPs also contribute to fluorescence, which overlaps with the spectrum of chlorophyll emission. In our studies, we have used PAM (Schreiber, 2004) and handy PEA for measuring chlorophyll *a* fluorescence.

5.1.5. Chlorophyll *a* fluorescence measurements using PAM fluorimeter

Changes in the yield of chlorophyll fluorescence were first observed as early as 1960 by Kautsky and co-workers (Kautsky et al., 1960). They found that, upon transferring photosynthetic material from the dark into the light, an increase in the yield of chlorophyll fluorescence occurred over a time period of around 1 s. This rise has subsequently been explained as a consequence of reduction of electron acceptors in the photosynthetic pathway, downstream of PSII, notably PQ and in particular, Q_A. Once PSII absorbs light and Q_A has accepted an electron, it is not able to accept another until it has passed the first onto a subsequent electron carrier (Q_B). During this period, the reaction centre is said to be 'closed'. At any point in time, the presence of a proportion of closed reaction centres leads to an overall reduction in the efficiency of photochemistry and so to a corresponding increase in the yield of fluorescence.

When a leaf is transferred from darkness into light, PSII reaction centres are progressively closed. This gives rise (during the first second or so of illumination) to an increase in the yield of chlorophyll fluorescence. Following on from this, however, the fluorescence level typically starts to fall again, over a time-scale of a few minutes. This phenomenon, termed fluorescence quenching, is explained in two ways. Firstly, there is an

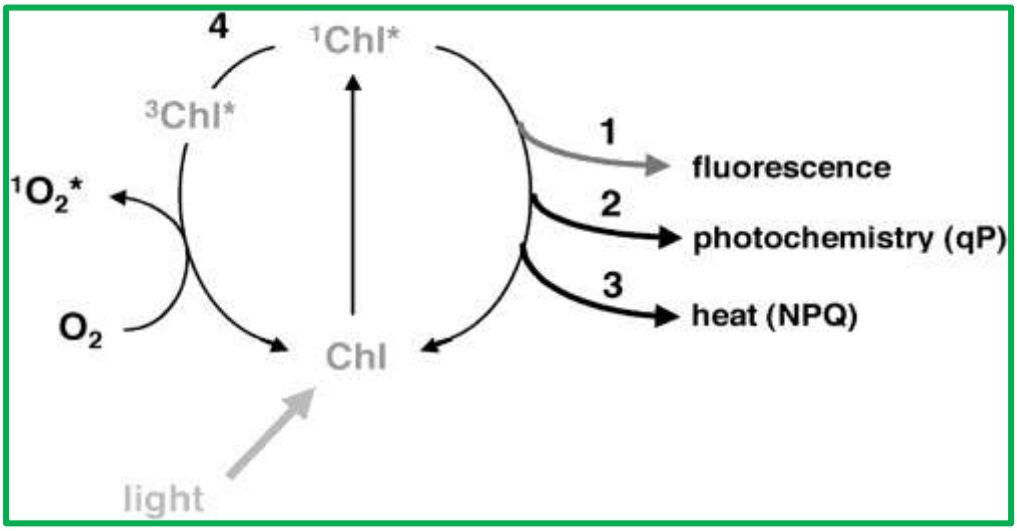


Fig.5.3. Possible fates of excited Chl. When Chl absorbs light it is excited from its ground state to its singlet excited state, ^{1}Chl*. From there it has several ways to relax back to the ground state. It can relax by emitting light, seen as fluorescence (1). Its excitation can be used to fuel photosynthetic reactions (2), or it can de-excite by dissipating heat (3); both of these mechanisms reduce the amount of fluorescence. They are therefore referred to as qP and NPQ of Chl fluorescence. Last,^{1}Chl* can, by intersystem crossing, produce^{3}Chl* (4), which in turn is able to produce$^1O_2$*, a very reactive oxygen species. (Adapted from Muller et al., 2001).

increase in the rate at which electrons are transported away from PSII; this is due mainly to the light-induced activation of enzymes involved in carbon metabolism and the opening of stomata. Such quenching is referred to as 'photochemical quenching'. At the same time, there is a increase in the efficiency with which energy is converted to heat. This latter process is termed 'non-photochemical quenching' (NPQ). In a typical plant, changes in these two processes will be complete within about 15–20 min and an approximate steady-state is attained, although the time taken to reach this state can vary significantly between plant species (Johnson et al., 1990).

The calculation of fluorescence parameters is explained by refering to a typical experimental trace (Fig.5.4). Measurement is initiated by switching on the weak measuring light, providing a measure of Fo (*upward arrow*). A saturating flash of light or multiple subsaturating flashlets that together trigger single turnover excitation (*filled arrow heads*), is then applied and allows the measurement of Fm from the dark-adapted sample. Next, actinic light (*open bar*) is applied and further saturating pulses are given at appropriate intervals to measure Fm′. The transient fluorescence, Ft, is monitored during the entire duration. The fluorescence minimum in the quenched state, Fo′, is measured immediately after the removal of actinic light (*downward arrow*). The variable fluorescence in the dark and in the quenched state (Fv′) is expressed by Fv.

5.1.6. Post illumination transients after light to dark transition

Typical induction transients in response to illumination by saturating light are shown, as measured with a standard PAM fluorometer equipped with a modified emitter-detector unit. Fluorescence was excited by modulated actinic light. In Figure 5.5, the responses of wild-type cells of *Synechocystis* PCC 6803 upon illumination with 8 min of saturating light are compared after turning off the saturating actinic light. In the wild-type the response is dominated by a rapid fluorescence increase which is followed by a dip and a slower rise

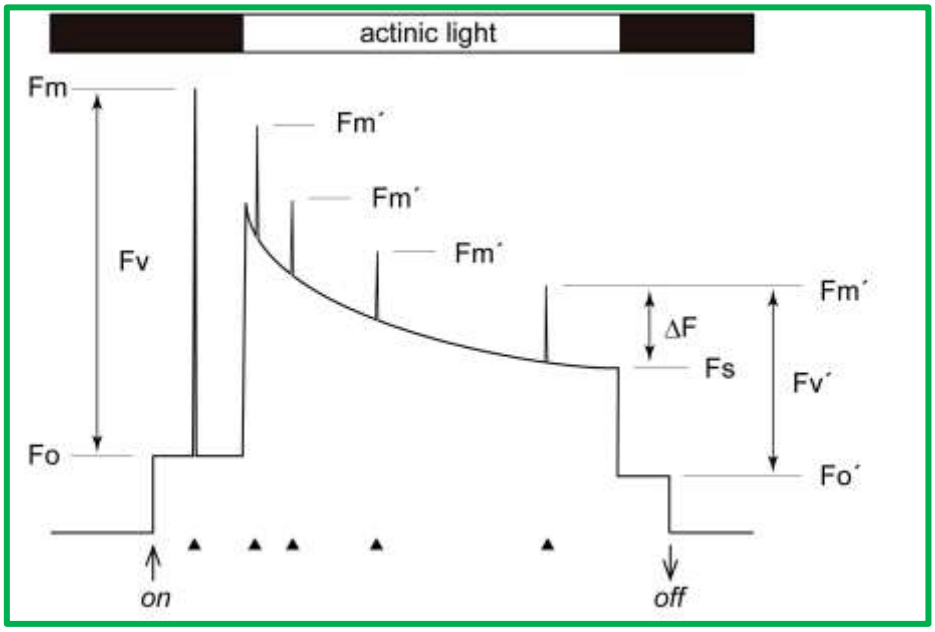


Fig .5.4. Parameters used in fluorescence quenching analysis. Calculation of fluorescence by referring to a typical experimental trace obtained with a PAM-type fluorometer. Measurement is initiated by switching on the weak measuring light, providing a measure of Fo (*upward arrow*). A saturating flash of light or multiple subsaturating flashlets that together trigger single turnover excitation (*filled arrowheads*), is then applied and allows the measurement of Fm from the dark-adapted sample. Next, actinic light (*open bar*) is applied and further saturating pulses are given at appropriate intervals to measure Fm′. The transient fluorescence, Ft, is monitored during the entire duration. The fluorescence minimum in the quenched state, Fo′, is measured immediately after the removal of actinic light (*downward arrow*). The variable fluorescence in the dark and in the quenched state (Fv′) is expressed by Fv = Fm – Fo, and Fv′ = Fm′ – Fo′, respectively.

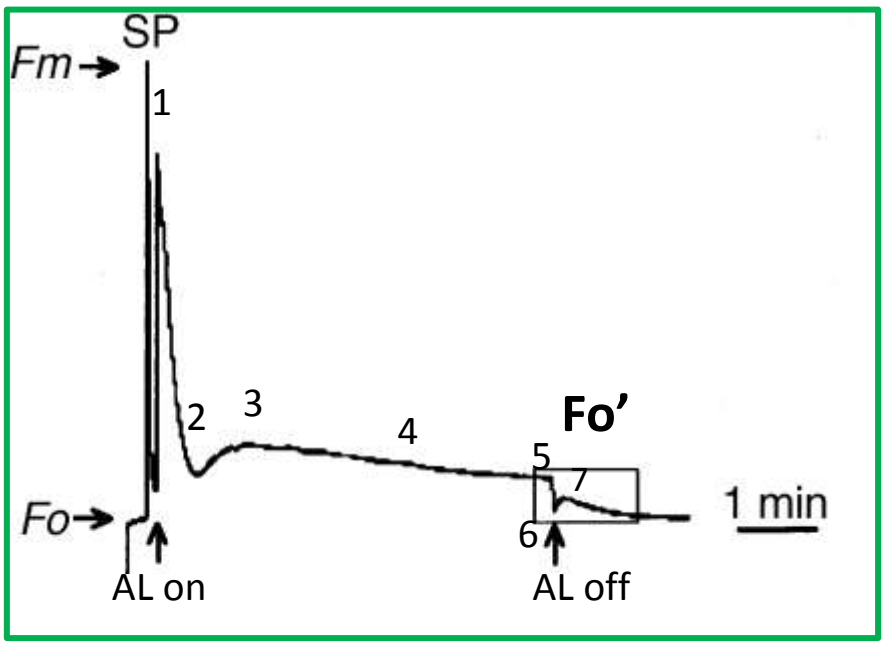


Fig.5.5. Typical chlorophyll *a* fluorescence transient of dark adapted cyanobacterial culture. Fluorescence transient were recorded during 8 min of actinic light (AL) and continued to record in absence of AL for 60 sec. Dark reduction of PQ pool was monitored when AL turned off. sp, saturation pulse . 1. First rapid rise 2. Dip phase. 3. Secondary rise phase .4. Stationary phase.5. Rapid decay phase. 6. Slow decay 7. Slow rise phase.

phase. Upon light-off there is a biphasic fluorescence decline leading to a level distinctly below the initial dark level (undershoot) followed by a slow rise back to the dark level.

Considering the complexity of the *in vivo* reactions involving NADP(H), at the present state of information an explanation of the various transients of the induction kinetics can be only tentative. However, based on available literature of photosynthetic reactions in wild-type the following interpretations appear reasonable. The changes are indicated with numbers in Fig.5.5.

1. First rapid rise: light driven reduction of NADP;
2. Dip phase: oxidation of NADP via Calvin cycle activity, which sets in as soon as the ribulose bisphosphate carboxylase is activated and ATP becomes available;
3. Secondary rise phase: accumulation of NADPH as its oxidation in the Calvin cycle becomes limited, possibly going along with a limitation in ATP-supply;
4. Stationary phase: matched rates of light-driven NADP reduction and NADPH oxidation via the Calvin cycle; electron storage in reduced carbon;
5. Rapid decay phase: dark-oxidation of NADPH via the Calvin cycle or alternative acceptor systems, as, for example, active oxygen species;
6. Slow decay phase: NADPH oxidation during oxidative phosphorylation;
7. Slow rise phase: dark-reduction of NADP associated with reductive pentose phosphate cycle.

These tentative interpretations shall serve as working hypothesis for the experiments presented in the following sections.

5.2. Results and Discussion

5.2.1. Δ*crhR* failed to operate light induced energy redistribution (State transitions) at low temperature

The slow growth and bleached phenotype appearence of the mutant strain under low temperature suggested that deletion of *crhR* could affect structural and functional aspects of photosynthetic apparatus. Chlorophyll fluorescence analysis could give more information about the structural and functional organization of major photosynthetic complexes and acclimation capacity of cells to temperature. Thus, the level of fluorescence can be changed by the state of the primary acceptor Q_A. When Q_A is oxidized, a minimal level of fluorescence can be observed, and a maximum level, when Q_A is fully reduced (Tsimilli et al., 2009). Fig.5.6 shows the fluorescence analysis of the wild-type and Δ*crhR* mutant cells before and after low temperature treatment at 24^{o}C for 72 h.

State transition as described earlier is a physiological adaptive mechanism that balances the distribution of light energy absorbed by the PBS between PSI and PSII in cyanobacteria (Murata, 1969; Mullineaux and Emlyn-Jones, 2005). According to the method of Ruban and co-workers, state transitions can be induced by switching on and off of PSI light (Far-red light) in the presence of continuous PSII light (actinic light). Maximal fluorescence levels in different states, (state 1 and 2 denoted as Fm^1 and Fm^2 respectively) can be probed by saturating light pulses (Kovacs et al., 2006). Although the transients upon PSI light on / off reflect the capacity of state transitions to balance the PQ redox state, Fm^1 and Fm^2 oscillations provide information on changes in the apparent PSII antenna cross section (Tsunoyama et al., 2009). The oscillations of Fm^1 and Fm^2 are due to association and dissociation of PBS with PSII and PSI. The increase in fluorescence in state 1 is due to association of PBS with PSII (Fm^1) and the decrease in fluorescence in state 2 (Fm^2) is due to dissociation of PBS from PSII and association with PSI. Switching on PSI light induces a

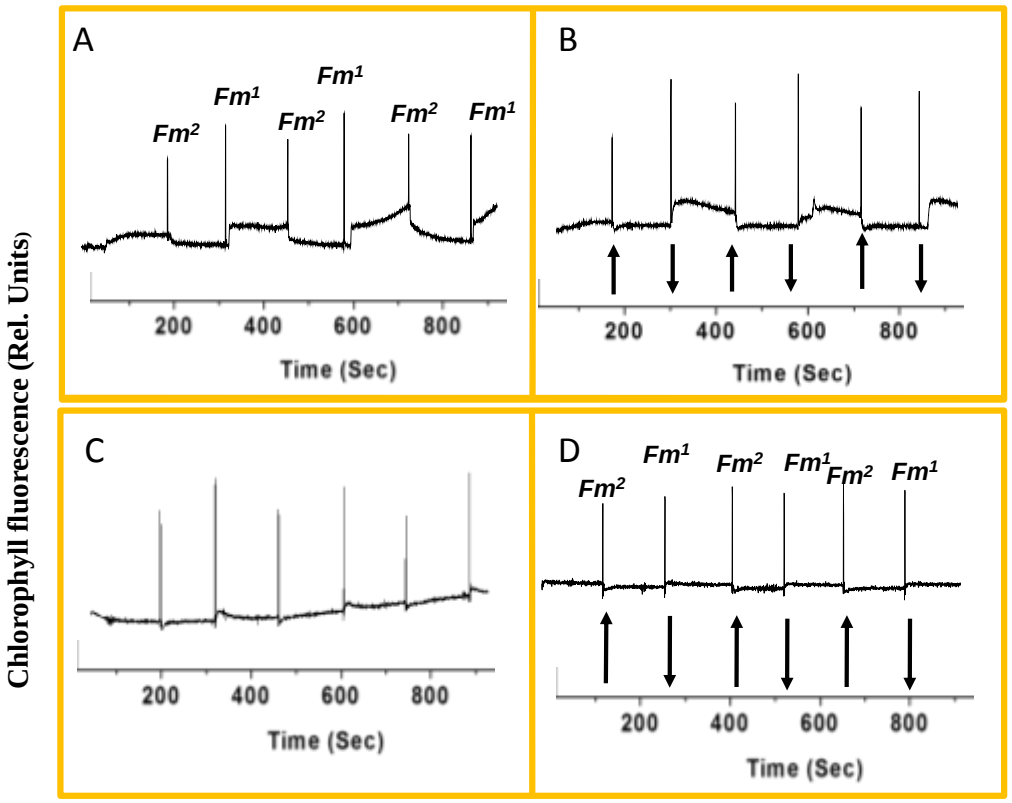


Fig. 5.6. State transitions examined by changes in the yield of chlorophyll fluorescence. (A) Wild-type cells grown at 34°C. (B) Wild type cells that had been grown at 34°C for 16 h and then incubated at 24°C for 72 h. (C) Δ*crhR* mutant cells grown at 34°C, (D) Δ*crhR* mutant cells that had been grown at 34°C for 16 h and then incubated at 24°C for 72 h. Saturated light pulse of 4000 µmol photons m^{-2} s^{-1} were given to record Fm in state 1 and state 2. Upward and downward arrows indicate "on" and "off" of far-red illumination. Actinic light of intensity 37 µmol photons m^{-2} s^{-1} was given.

gradual increase in chlorophyll fluorescence reflecting an increasing PSII antenna cross section during state 2 to state 1 transition (Kovacs et al., 2006). Switching off PSI light with actinic light on causes a rapid transient raise that was followed by decay to the original level. This indicates a temporary increase in the PQ reduction level (absence of PSI oxidation power), which was followed by PQ reoxidation and a decrease of PSII antenna cross section (state 1 to state 2 transition). Changes in the apparent antenna cross section can be visualised by saturating light pulses after and before far-red periods, revealing, Fm^2 and Fm^1 respectively. The Fm^1 and Fm^2 oscillations (with far-red on and off respectively) were more pronounced in the wild-type and Δ*crhR* cells grown at 34^{o}C (Fig.5.6A and B). Wild-type cells shifted to 24^{o}C for 72 h also exhibited the mechanism of energy distribution though little variations were observed compared to wild-type cells grown at 34^{o}C (Fig.5.6C). This indicates significant transient changes in the PQ redox level that inturn induce a more robust change in the PSII antenna cross section. However, results presented in Fig.5.6D shows that the Δ*crhR* mutant cells failed to exhibit state transitions when the cells shifted to 24^{o}C for 72 h. Neither significant transient changes nor changes in the antenna cross section were induced by switching on/ off far-red illumination with actinic light on in the Δ*crhR* mutant cells (Fig.5.6D). Inability to perform state transitions in the Δ*crhR* mutant indicates probably due to the changes in PQ oxidation/reduction state. Hence, we further investigated the redox state of the PQ pool under low temperature condition in the wild-type and Δ*crhR* mutant cells by using post illumination chlorophyll *a* fluorescence induction curves.

5.2.2. Redox state of PQ pool in the wild-type and Δ*crhR* at low temperature

5.2.2.1. Post illumination transients using PAM fluorometry

Using a PAM fluorometer, the electron flow can be monitored *in vivo* by a change in chlorophyll fluorescence. In particular, the electron flow from NADPH to the intersystem chain can be roughly estimated by transient increase in chlorophyll fluorescence after a

period of illumination with actinic light (Schreiber et al., 1986). The fact that far-red light, which activates predominantly PSI photochemistry, is able to quench this increase in fluorescence demonstrates that NADPH transfers electrons to the PQ pool (Shikanai et al., 1998).

Cyanobacterial cells are usually in state 2 due to respiratory electron transport in dark. The apparent rise in Fo (Chlorophyll fluorescence increase) after a light to dark transition was used as a measure of dark reduction of the PQ pool by respiratory reductants and referred as Fo′ (Mano et al., 1995). The cultures were exposed with actinic light for 8 min and then turned off to monitor the fluorescence yield changes in the dark. Any rise in fluorescence is denoted as Fo ′. A transient rise in Fo′ level over a period of 20 sec after turning off the actinic light was detected in the wild-type and Δ*crhR* cells when grown at 34^{o}C (Fig.5.7). Such Fo′ increase can be attributed to respiratory electron flow to the PQ pool in the dark and/or the back flow of electrons due to PSI cyclic electron transport (Campbell et al., 1998). In order to know whether the reduction of PQ pool under darkness is due to respiratory electron flow, we blocked the respiration by addition of $HgCl_2$ and measured the Fo′ (Fig. 5.7).

$HgCl_2$ is known to inhibit numerous enzyme reactions. It was also found to affect NDH-mediated cyclic electron flow around PSI (Mi et al., 1992a; Mi et al., 1992b). As shown in Figure 5.7, the relatively low concentration of 40 μM $HgCl_2$ has a dramatic effect on the apparent changes in NADPH upon illumination, in the wild-type cells. Notably, the initial positive responses of the controls are transformed by $HgCl_2$ into negative transients, thus addition of $HgCl_2$ completely eliminated the Fo′ under low temperature in the wild-type cells, confirming the dark induced enhancement of Fo′ under low temperature in the wild-type is due to respiratory electron flow suggesting that NADPH is oxidized in a rapid light-driven reaction.

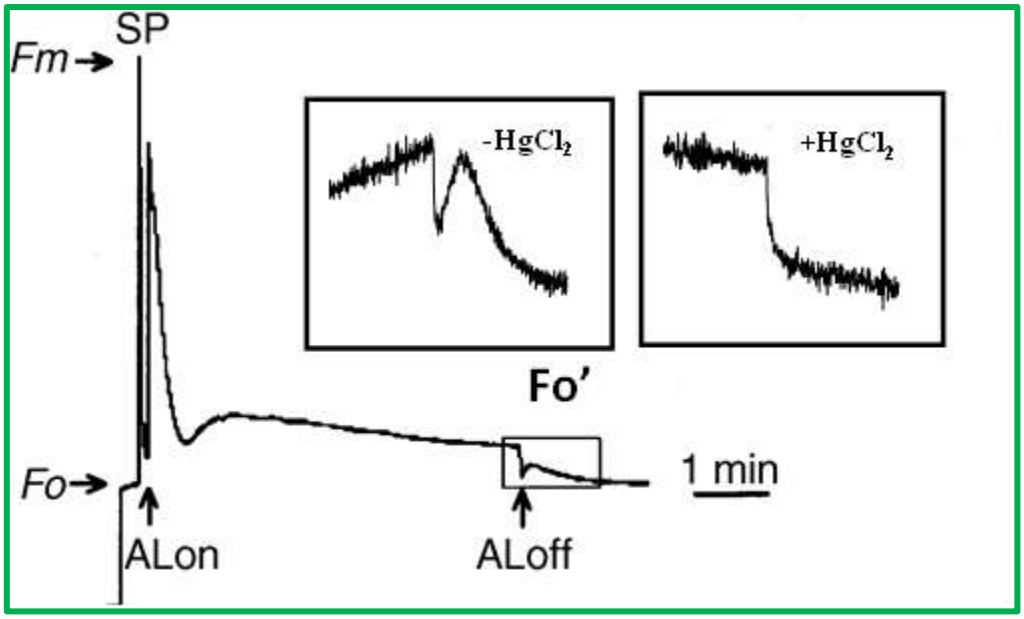


Fig.5.7. Post illumination transients from steady state fluorescence to Fo′. After the actinic light (AL; 37 µmol photons m^{-2} s^{-1}) was turned off in wild-type cells grown at 34°C and treated with $HgCl_2$. Changes in the Fo′ were monitored; Inset indicates the presence and absence of $HgCl_2$ (represented in boxes)

5.2.2.2. Δ*crhR* cells are locked in state 1 with oxidized PQ pool

Dark-adapted cyanobacterial cells are usually in state 2 due to respiratory electron transport, which is characterized by low fluorescence yield of PSII (Mullineaux and Allen, 1990). A transient rise in Fo′ level over a period of 20 sec. after turning off the actinic light was detected in the wild-type and Δ*crhR* cells when grown at 34^{o}C (Fig.5.8A and B). Such Fo′ increase can be attributed to respiratory electron flow to the PQ pool in the dark and/or the back flow of electrons due to PSI cyclic electron transport (Campbell et al., 1998). Whereas incubation of wild-type cells at 24^{o}C for 72 h, exhibited a significant raise in Fo′, indicating higher degree of PQ pool reduction under low temperature in the wild-type cells (Fig.5.8C). Since photosynthetic and respiratory electron transfer chains of cyanobacteria share common redox components such as PQ (Scherer, 1990), it is generally accepted that the dark reduction of PQ pool could be due to respiratory electron flow (Mullineaux and Allen, 1986, 1990). Low temperature induced enhancement in Fo′ may be due to the change in the membrane fluidity, thereby enhanced transfer of electrons from respiratory reductants to PQ pool. Δ*crhR* cells incubated at 24^{o}C for 72 h did not show any raise in Fo′ suggesting inefficient electron flow from respiratory chain (Fig.5.8D).

5.2.2.3. Oxidation of the PQ pool by illuminating with far red light

Illumination with far-red background light which preferentially excites PSI and thus drives oxidation of PQ pool, completely eliminated the post illuminated increase of Fo′ in both wild-type and Δ*crhR* cells grown at 34^{o}C (Fig.5.9A and B). Switching on the far-red light, could not completely reoxidize the reduced PQ pool at low temperature in the wild-type cells, indicating majority of PQs in PQ pool are in a highly reduced condition under low temperature (Fig.5.9C). However, Δ*crhR* cells incubated at 24^{o}C for 72 h did not show any raise in Fo′ suggesting in efficient electron flow from respiratory chain (Fig.5.9D). Taken together, the PQ pool is kept under highly reduced state in the wild-type cells when incubated

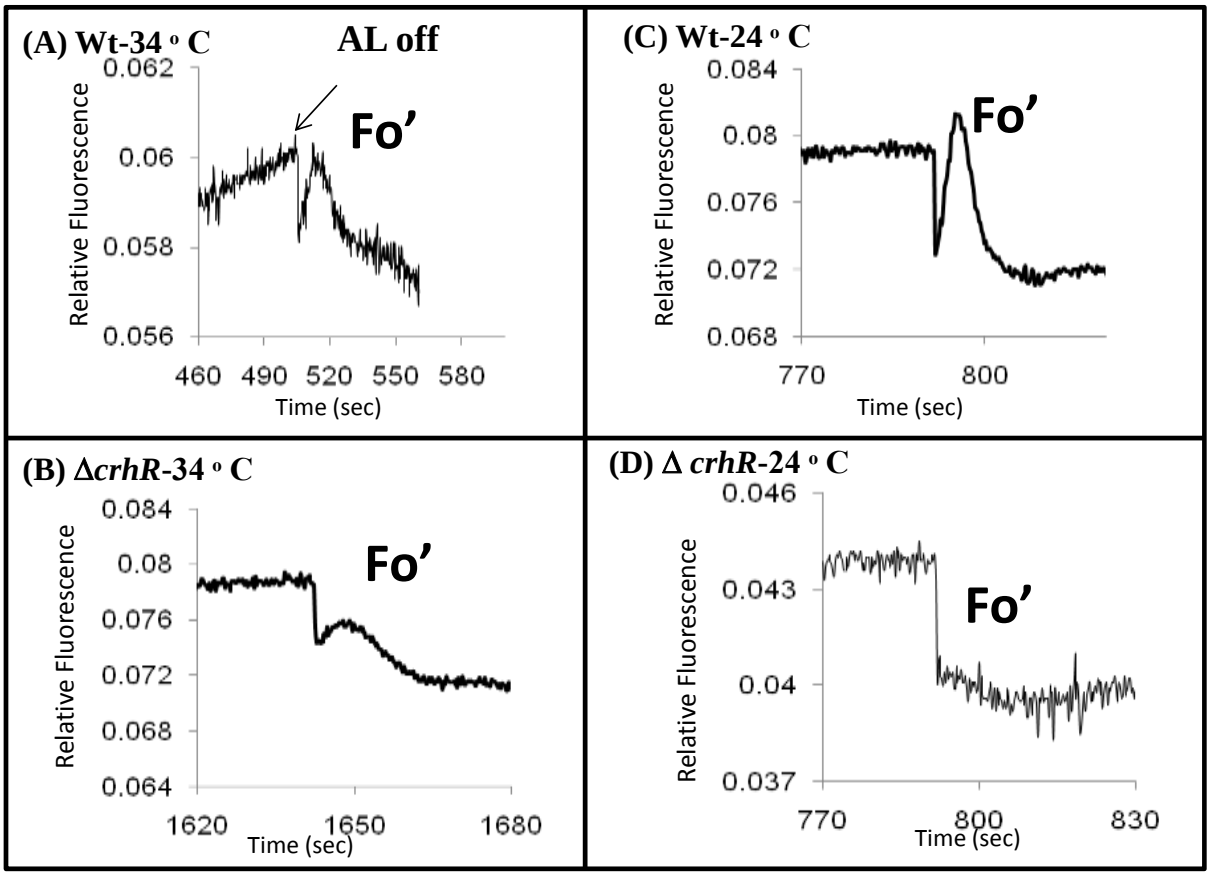


Fig.5.8. Post illumination transients from steady state fluorescence to Fo′ after the actinic light (AL; 37 µmol photons m^{-2} s^{-1}) was turned off to determine the dark reduction of PQ pool in wild-type cells and Δ*crhR* cells grown at 34^{o}C for 16 h (A and B) and wild-type cells and Δ*crhR* mutant cells cells grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h respectively (C and D).

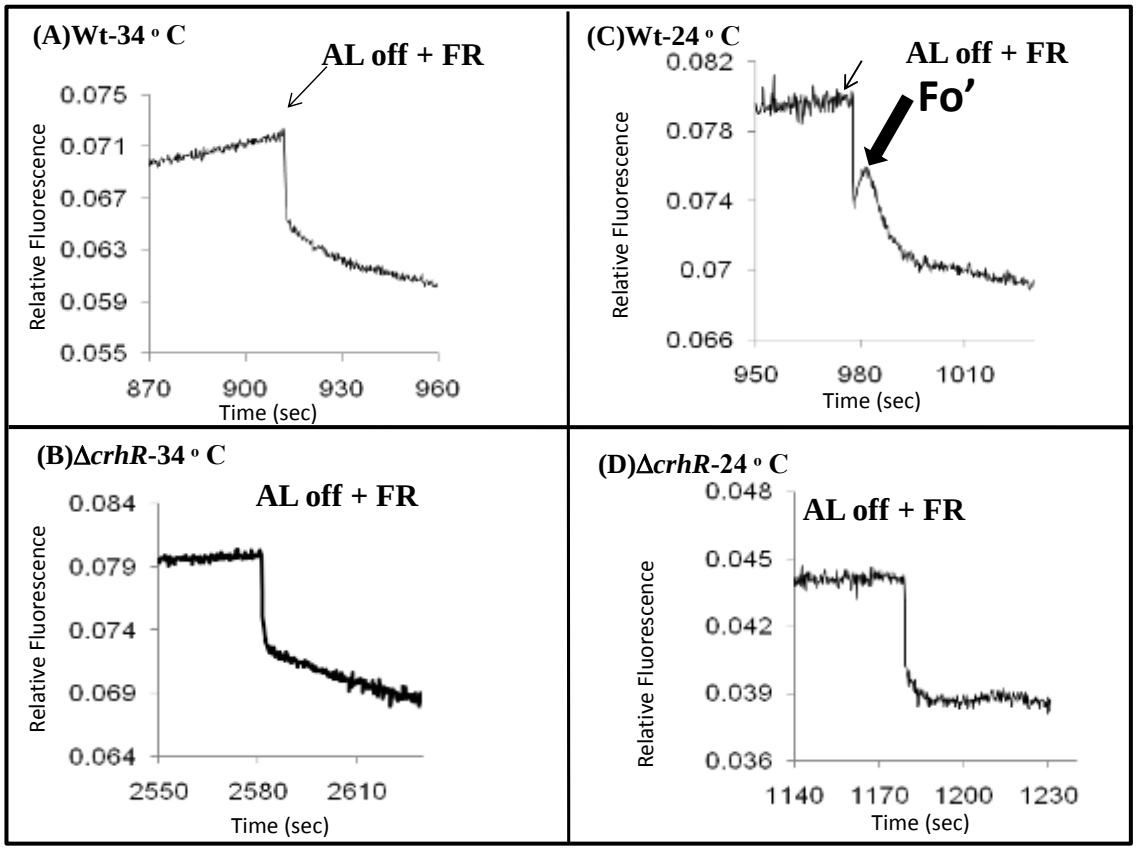


Fig.5.9. Chl *a* post illumination transients for determination of reoxidation of PQ pool by turning off AL and turning on the FR in wild-type cells and Δ*crhR* cells grown at 34^{o}C(A and B) wild type cells and Δ*crhR* cells grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h respectively(C and D). The intensity of far-red light (FR) applied after turning off the actinic light (AL) was 10 W m^{-2}.

at 24^{o}C for 72 h. In contrast, in Δ*crhR* cells the PQ pool was kept under oxidized state.

5.2.3. Prominent S to M rise in Δ*crhR* -24^{o}C confirms that Δ*crhR* cells are in state 1 at low temperature

State transitions have been visualized to involve direct transfers from PBS to PSII and PSI (namely, PSII←PBS→PSI), indirect transfer to PSI via PSII (known as spillover; PBS→PSII→PSI) or both routes (hybridmodel; McConell et al., 2002). Basically, the regulation is effected via the PBS→PSI excitation transfer route: when it is maximized, the PBS-sensitized chlorophyll *a* fluorescence is low, i.e., the cells are in state 2; when it is minimized, the PBS→PSII route is enhanced and the PBS-sensitized chlorophyll *a* fluorescence is high, i.e., the cells are in state 1. At room temperature, the conversion of photosynthetic cells from state 2 to state 1 is observed by a kinetic rise of chlorophyll *a* fluorescence, whereas the state 1 to 2 conversion by a decline of chlorophyll *a* fluorescence.

5.2.3.1. Slow fluorescence transients in *Synechocystis* wild-type and Δ*crhR* before and after low temperature

The regulation of electronic excitation and its distribution are reflected in FI (Fluorescence induction) pattern usually measured in the 690–700 nm spectral range that relates to changes in PSII photochemistry. The fast part of FI curve in cyanobacteria (first second of actinic irradiance) is rather shallow in comparison to the well-structured and dynamic OJIP kinetics in higher plant leaves (Papageorgiou et al., 2007). The fast OJIPS transient is followed by a much slower fluorescence increase from the S-plateau to the M peak appearing tens of seconds after the onset of light. The SM rise of FI is dominant in cyanobacteria in contrast to the higher plants and green algae(Fig.5.10) (Papageorgiou et al., 2011) and has been assigned mostly to an increased PBS → PSII excitation transfer for the following reasons: (i) it has been observed only in PBS-sensitized chlorophyll *a* fluorescence (Papageorgiou et al., 2007) ; (ii) it is usually much lower or is absent in higher plants, green algae and non-PBS

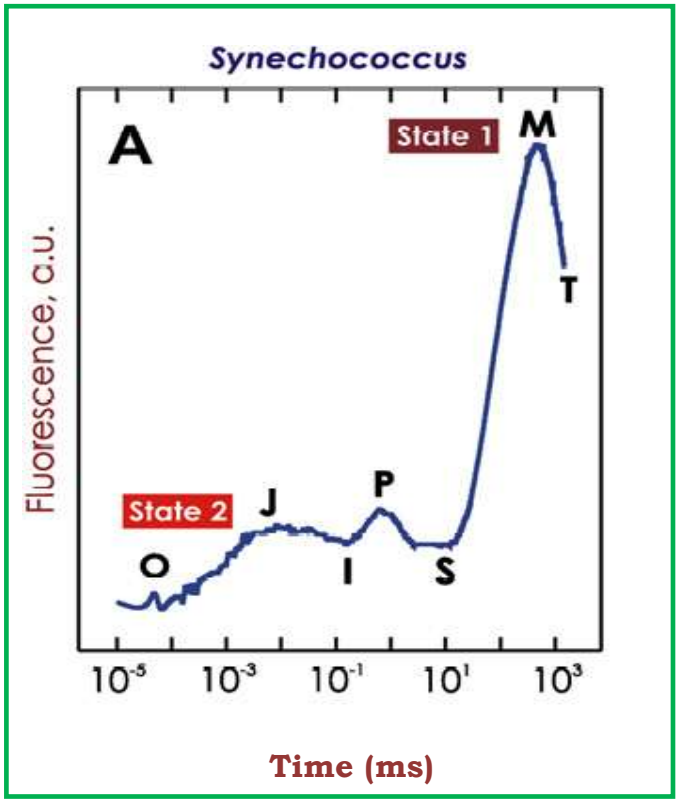


Fig.5.10. State transitions in the PBS containing cyanobacterium *Synechococcus* as detected by room temperature fluorometry. After dark adaptation, *Synechococcus* exists essentially in state 2 (see P level) and upon intense (red) actinic light illumination (3000 μmol photons m^{-2} s^{-1}) it shifts essentially to state 1 (levels M, T). Adapted from Papageorgiou,2011.

containing cyanobacteria (Papageorgiou et al., 2007) (iii) hyper-osmotic conditions block the SM rise in PBS-containing cyanobacteria reversibly, without blocking the OJIPS phase (Stamatakis et al.,2007); (iii) the SM rise can be observed also in the presence of DCMU (diuron, that blocks electron transfer from PSII to PSI), and this rules out the involvement of Q_B and the PQ-pool in it (Tsimilli et al., 2009). Blocking of the PBS → PSI excitation transfer enhances the SM rise (maximal PBS excitation ends up in PSII) and on the other hand stimulation of this transfer can fully abolish the SM rise as much less PBS excitation ends up in PSII (Papageorgiou et al., 2007; Stamatakis et al., 2007) . This phenomenology suggests that the SM rise reflects the regulatory distribution of PBS excitation, which is known as state 2-to-state 1 transition (Mullineaux and Jones, 2005),since cyanobacteria tend to stay in the low fluorescence state 2 during dark and are transformed into the highly fluorescent state 1 during irradiation (Stamatakis et al., 2007).

The fast OJIPS transient is followed by a much slower fluorescence increase from S to M appearing after ten seconds upon onset of light in *Synechocystis* (Fig.5.11). Such a prominent S to M rise has not been observed in the dark adapted cyanobacterial cells (cells in state 2) (Fig.5.11). Thus prominent S to M rise in the fluorescent transient indicates that the cyanobacterial cells are in state 1 condition.

Chlorophyll *a* fluorescence transient measurements were used to assess the state of the wild-type and Δ*crhR* cells at low temperature. S to M rise in the fluorescent transients of WT-34^{o}C-cells, WT-24^{o}C-cells and Δ*crhR*-34^{o}C-cells was not observed, suggesting that these cells are in state 2. In contrast, Δ*crhR*-24^{o}C-cells exhibited a prominent S to M rise, indicating that low temperature incubation of Δ*crhR* cells induce state 2 to state 1 transition(Fig.5.12).

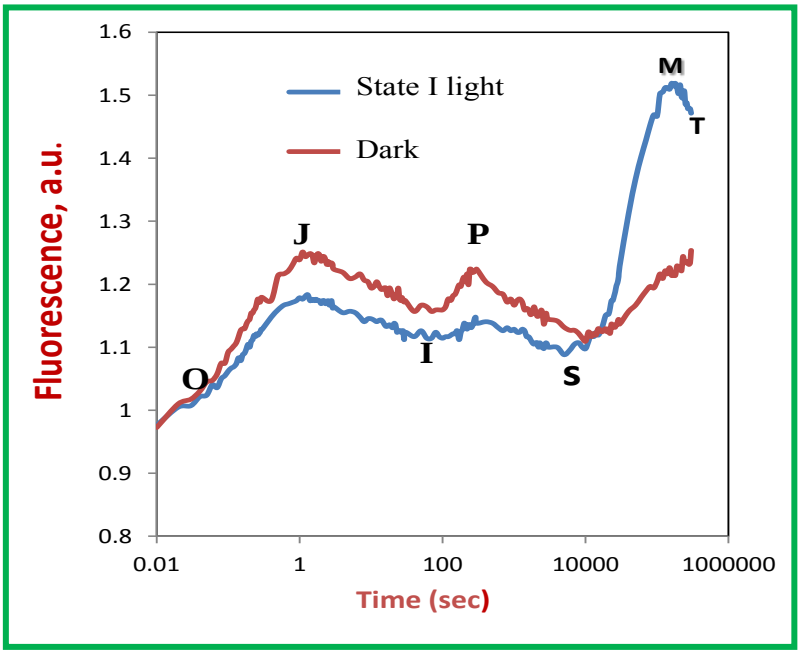


Fig.5.11. The fast OJIPS Chl fluorescence transient is followed by a much slower fluorescence increase from S to M in *Synechocystis* after the onset of light. OJIPSMT transient of dark incubated *Synechocystis* cells is indicated in red. The fast OJIP followed by an increase from S to M rise was monitored after illuminating the cells with state 1 light which is indicated in blue.

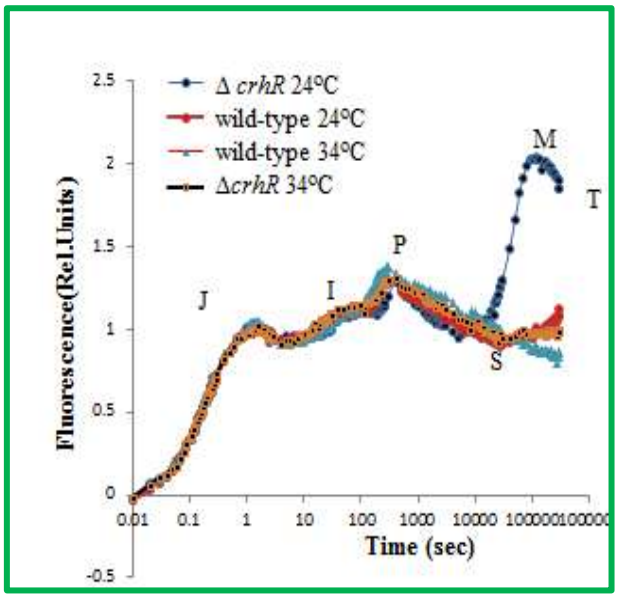


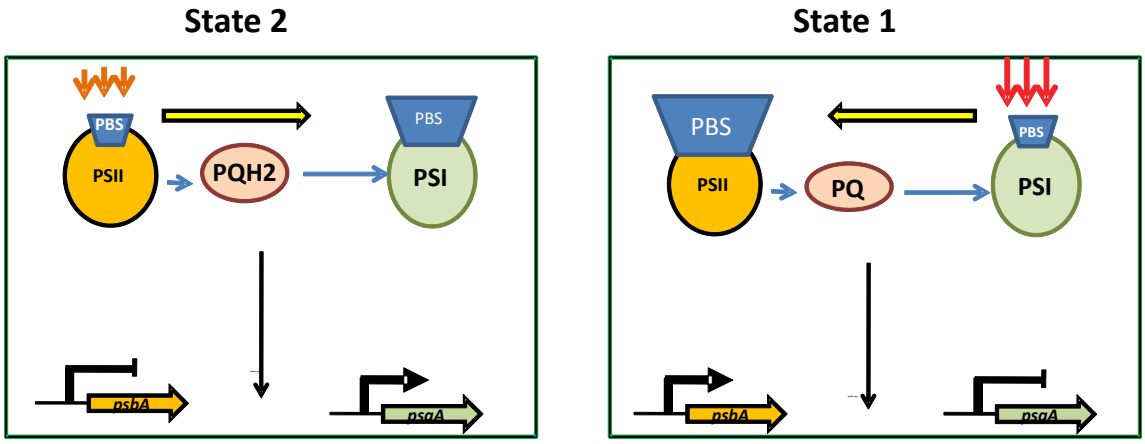
Fig.5.12. The fast OJIPSMT Chlorophyll *a* transient from wild-type and Δ*crhR* cells at 34^{o}C and 24^{o} C. Δ*crhR*-24^{o}C-cells exhibited S to M rise which is a characteristic feature of state 2 to state 1 transition. Spectras were normalised at F5 for better comparision of the state differences. These fluorescence kinetic measurements were made using a portable handy PEA fluorimeter.

5.3. Conclusion

In the process of acclimation to 24^{o}C, wild-type cells exhibited a response in which the PQ pool tends to be in reduced state. The enhanced reduction of PQ pool is due to acceleration of electrons from respiratory reductants and may also due to increased cyclic electron flow. The reduced state of PQ pool is a signal for fine tuning of photosystem stoichiometry for low temperature acclimation. However, we observed that the Δ*crhR* cells at 24^{o}C shifted permanently to state 1 condition. Such mechanism was reported as "State shift" (Mohanty et al., 2011). The wild-type cells (24^{o}C adapted) exposed to far red light (oxidizes), were able to exhibit state transitions. Whereas, Δ*crhR* cells were unable to exhibit acclimation to 24^{o}C and the cells were arrested in state 1 and finally failed to exhibit state transition.

Role of RNA helicase in regulation of low temperature inducible gene expression for optimized photosynthesis

Chapter 6



6.1. Introduction

6.1.1. Photosystem stoichiometry

Energy transduction in photosynthesis depends on the coordination of two photosystems, PSII and PSI. PSII conducts a light-dependent oxidation of water and reduction of PQ (Barber, 2002; Diner and Rappaport, 2002), while PSI is involved in a light-dependent electron transport from plastocyanin to $NADP^+$ via ferredoxin (Fromme et al., 2001). The two light-dependent reactions are connected by cytochrome b_6/f complex that catalyzes the electron transfer from PQ to plastocyanin. Since PSII and PSI are functioning in tandem in photosynthetic electron flow, photosynthetic organisms must maintain the balance of two photosystems. PSII and PSI have respective light harvesting complexes with different pigment composition, thus absorbing light of different wavelengths. The size of the light-harvesting antennae in each photosystem is also altered, depending on photon flux densities of the environments. Thus, photosynthetic organisms must modulate photosystem stoichiometry, i.e. PSI/PSII ratio, in response to environmental changes.

When redistribution of energy via state transitions is insufficient to maintain the poise in light energy distribution between PSII and PSI, long-term adjustments at the level of PSII/PSI stoichiometry occur. Like state transitions, photosystem stoichiometry adjustment is an acclimatory response to changes in light quality, but it is long term in scope, taking hours and days to complete instead of minutes and it can also result in larger adjustments in light-harvesting antenna size. This modulation in the ratio of the photosystems appears to occur primarily at the level of PSI reaction center polypeptides (Aizawa and Fujita, 1992; Fujita, 1988; Murakami et al., 1997b; Pfannschmidt et al., 2001; Tullberg et al., 2000). It was first proposed that modulation of photosystem stoichiometry is a response to changes in the redox state of the intersystem electron transport chain to ensure equal rates of electron flow through both PSI and PSII (Allen and Pfannschmidt, 2000). Though it was considered that

photosystem stoichiometry adjustments involve regulation of *de novo* synthesis of chlorophyll *a* and chlorophyll *a*-binding proteins as precursors to new photosystems (Murakami et al., 1997), how this was done remained unknown for nearly a decade. By using photosystem-specific light conditions and electron transport inhibitors that limit the rate of photosynthetic electron transport at specific points of the electron transport pathway, it was found that the redox state of PQ pool, the point of regulatory control of the LHCII kinase, also controls the transcription of chloroplast genes that encode the core reaction centre apoproteins of the photosystems (Pfannschmidt et al., 1999; Tullberg et al., 2000; Puthiyaveetil and Allen, 2008). This PQ-regulated chloroplast reaction centre gene transcription is the initiation step of the overall molecular mechanism of photosystem stoichiometry adjustment. In PSI light, the PQ pool becomes more oxidized, repressing PSI gene transcription and/or activating PSII gene transcription. In the complementary PSII light conditions, the transcription of PSI genes is activated and/or PSII genes are repressed. The demonstration stated that state transitions and photosystem stoichiometry adjustment are controlled by the same regulatory signal -PQ pool redox state- revealed the analogous nature of these two responses (Fig.6.1). Thus State transitions operate primarily at the post-translational level and stoichiometry adjustments at the transcriptional level of gene regulation, with both responses serving to compensate for imbalance in excitation energy caused by changes in the quality of light (Allen, 1992, 1995; Allen and Pfannschmidt, 2000; Pfannschmidt, 2003; Rochaix, 2011).

6.1.2. Studies on dynamics of Photosystem stoichiometry

The abundance of PSI, PSII and Cyt b_6f complexes was studied in the cells of *Synechocystis* PCC 6714 grown under various conditions (Fujita and Murakami, 1987). The stoichiometry of PSI to PSII varied markedly depending on light quality, but the ratio of PSII to Cyt b_6f remained fairly constant. Furthermore, the PBS amount, as indicated by PC and APC,

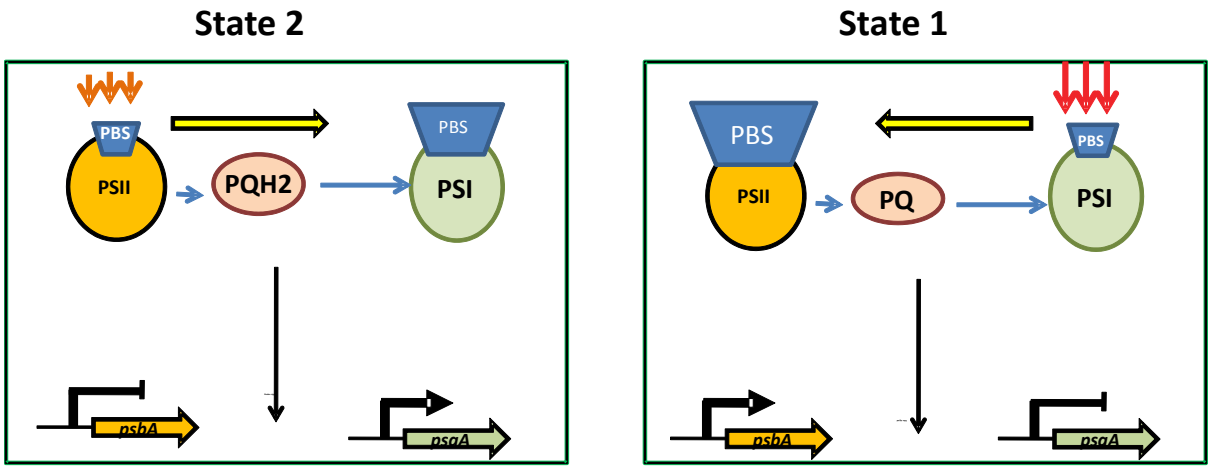


Fig.6.1. Proposed model of state transitions for the present study. State 1 is achieved by oxidation of intersystem electron carriers (usually by "excess" excitation of PSI). Reduction of intersystem electron carriers, most likely PQ, either by "excess" excitation of PSII or by a dark respiratory pathway, triggers the conversion to state 2. State 2 is characterized by a decrease in PSII variable fluorescence, a decrease in the PSII absorbance cross section, and an increase in the PSI absorbance cross section as compared with state 1.

relative to PSII was also constant under autotrophic conditions. The stoichiometry in cells that were heterotrophically grown was almost the same as that in cells grown under PSII light. From these results, it was concluded that the variable component responsible to the variation of photosystem stoichiometry is PSI (Fujita and Murakami, 1987). Later, similar kind of observations were made with *Synechocystis* PCC 6803 (Aizawa and Fujita, 1992). Based on such results a hypothesis was proposed that the variation is induced in response to redox state of the electron pool between the two photosystems (Fujita et al., 1987).

In cyanobacteria, PSI/PSII ratio is normally about 2 and ranges from 1 to 4 depending on the light condition (Melis and Brown, 1980). Light that excites mainly PSII leads to the increase in PSI so as to bring about high PSI/PSII ratio of about 2 to 4, while light that excites mainly PSI induces the decrease in PSI, resulting in low PSI/PSII ratio as low as 1 (Fujita et al., 1985, 1988; Fujita and Murakami, 1987). The regulation of photosystem amounts by light quality is at the transcriptional and/or posttranscriptional level of PSI and PSII subunits (Glick et al., 1986).

A well-documented acclimation response is the adjustment of photosystem stoichiometry, i.e. the ratio of PSI to PSII was observed widely in cyanobacteria (Kawamura et al., 1979, Fujita et al., 1985, Manodori and Melis, 1986; Hihara et al., 1998), algae (Melis et al., 1996) and higher plants (Chow et al., 1990). Such adjustment has been suggested to balance the excitation of the two photosystems, containing different light harvesting antennae, under various wavelengths of light (Manodori and Melis 1986; Murakami and Fujita, 1991), or to protect the cells from photodamage under high irradiances (Hihara et al., 1998; Sonoike et al., 2001). In cyanobacteria, the changes in the PSI/PSII ratio have been shown to result mainly from a specific regulation of PSI abundance (Fujita et al., 1985; Aizawa et al., 1992; Fujita, 1997; Hihara et al., 1998). Although the structure and function of PSI are relatively well defined (Fromme et al., 2001), much less is known about the

mechanisms regulating the biogenesis and acclimation of this multiprotein complex. In cyanobacteria, regulation of PSI synthesis during light acclimation has been suggested to occur mainly at the level of translation or assembly of the complex (Aizawa and Fujita, 1997; Fujita, 1997). Analyses of cyanobacterial mutants with an aberrant PSI/PSII ratio have indeed several protein factors specifically involved in stable accumulation of PSI complexes (Wilde et al., 1995; Bartsevich and Pakrasi, 1997; Mann et al., 2000; Wilde et al., 2001; Shen et al., 2002), and the availability of chlorophyll *a* has also been considered as a regulatory factor due to selective suppression of PSI accumulation by inhibitors of chlorophyll *a* biosynthesis (Fujita et al., 1990). Although required for accumulation of stable PSI complexes, the significance of these factors in regulation of PSI content has not been established as yet. Thus far, the only protein unequivocally shown to regulate the PSI content during light acclimation is PmgA, which is required to suppress the accumulation of PSI complexes under long-term high-light stress (Hihara et al., 1998). Transcriptional regulation of PSI accumulation, on the other hand, was long neglected in cyanobacteria. Recently, a number of genome-wide microarray analyses of *Synechocystis* sp. PCC 6803 have revealed notable changes in the abundance of transcripts encoding subunits of PSI under various environmental conditions, such as elevated light intensity (Hihara et al., 2001), low temperature (Suzuki et al., 2001), UV-B light (Huang et al., 2002) or low CO_2 pressure (Wang et al., 2004). Furthermore, it has been shown previously by Northern blot analyses that the inhibition of the photosynthetic electron transfer chain by chemicals prevents the light-induced accumulation of psaE transcripts (Alfonso et al., 2000). The PmgA-dependent suppression of PSI content was later shown to result from transcriptional repression of PSI reaction center genes after prolonged high-light illumination (Muramatsu and Hihara ,2003), although the PmgA protein did not appear to regulate the early events of high-light acclimation (Hihara et al., 1998, Muramatsu and Hihara 2003). Regulation of the monomer

and trimer contents (chapter 4) allows to change the absolute amount of PSI and its ratio to that of PSII in the thylakoid membrane, the physiological significance of low temperature stress responses that adjusts the photosystem stoichiometry has not been fully established.

In order to get a deeper insight into the regulation of PSI synthesis during the initial stages of low temperature acclimation, we studied the expression and regulation of the *psaAB* operon, encoding the essential PSI reaction center proteins PsaA and PsaB, upon exposure of wild-type *Synechocystis* sp. PCC 6803 and Δ*crhR* mutant to low temperauture.

6.2. Results and Discussion

6.2.1. Effect of low temperature on photosystems

As discussed in the chapter 4, (Fig.4.4) fractionation of thylakoid membranes isolated from low temperature incubated Δ*crhR* cells also showed a significant decrease in the PSI trimer and an increase in the PSII/PSI monomer fraction as compared to the Δ*crhR* untreated cells (Fig.6.2). The extent of changes in photosystems due to low temperature in the *Synechocystis* wild-type and Δ*crhR* cells was further monitored by 77K analyses to further monitor the changes in the photosystems.

6.2.2. Low temperature leads to changes in Photosystem stoichiometry

Separation of photosynthetic pigment complexes on equal protein basis indicated a significant loss of PSI trimer in Δ*crhR* (Fig.4.5). Sucrose gradient ultra-centrifugation further allowed us to examine the 77K fluorescence emission spectra of pigment complexes in more detail (Fig.6.2 and Fig.6.3).

6.2.2.1. Photosystem stoichiometry changes by 77K fluorescence spectral analysis

77K fluorescence is a useful tool for analysing photosynthetic energy transfer between pigments and the distribution of energy between the PSI and PSII (Weis, 1985). In contrast to room temperature fluorescence, 77K fluorescence can distinguish the energy transfer of the PBP bound to the photosynthetic machinery, as well as chlorophyll *a* fluorescence emissions

from PSI and PSII. The excitation 436 nm wavelength leads to emission at the peak at 685 nm which is originated mainly from PSII-associated chlorophyll and peak at 720 nm is from the chlorophyll of PSI. Fig.6.2 shows the typical fluorescence emission spectra of F2 (PSII and PSI monomer mixture) of the wild-type cells grown at 34^{o}C and the wild-type cells incubated at 24^{o}C for 72 h after excitation at 436 nm. Raise in F720 emission peak in the wild-type-24^{o}C cells when compared with that of wild-type -34^{o}C cells, suggests enhanced PSI monomer levels due to low temperature treatment. This suggests that a fraction of PSI trimers were converted into monomers during incubation of wild-type cells at low temperature. In contrast, F2 of low temperature treated Δ*crhR* cells showed a significant decrease in the F685 peak and an increase in F720 fluorescence peak indicating a drastic decrease in the PSII content (Fig.6.2). This decline in the content of PSII reflects the loss of photosynthetic activity under low temperature in Δ*crhR* cells.

77K fluorescence emission spectra of the sucrose density gradients indicate that the PSII/PSI ratio was found to be altered under low temperature stress conditions in the wild-type indicating that this alteration is absolutely necessary for the cells to modulate themselves to the changed environment. However such alteration of the PSII/PSI ratio seemed to be changed drastically in Δ*crhR* supporting the inability of the cells to cope up the low temperature stress which is further preceded by the decrease in PSII levels (Fig.6.2).

6.2.3. Low temperature leads to the reduced content of PSI trimer Δ*crhR*

Excitation of F3 at 436 nm and monitoring the 77K fluorescence emission spectra revealed a major peak at 720 nm, which corresponds to PSI-associated chlorophyll. There was no significant change in the F720 fluorescence emission peak of F3 between of wild-type cells grown at 34^{o}C and wild-type cells that had been grown at 24^{o}C for 72 h (Fig.6.3). In addition, we noticed a decrease in PSI trimer content due to low temperature treatment in the Δ*crhR* cells (Fig.6.3). In contrast, F3 of low temperature treated Δ*crhR* cells showed a

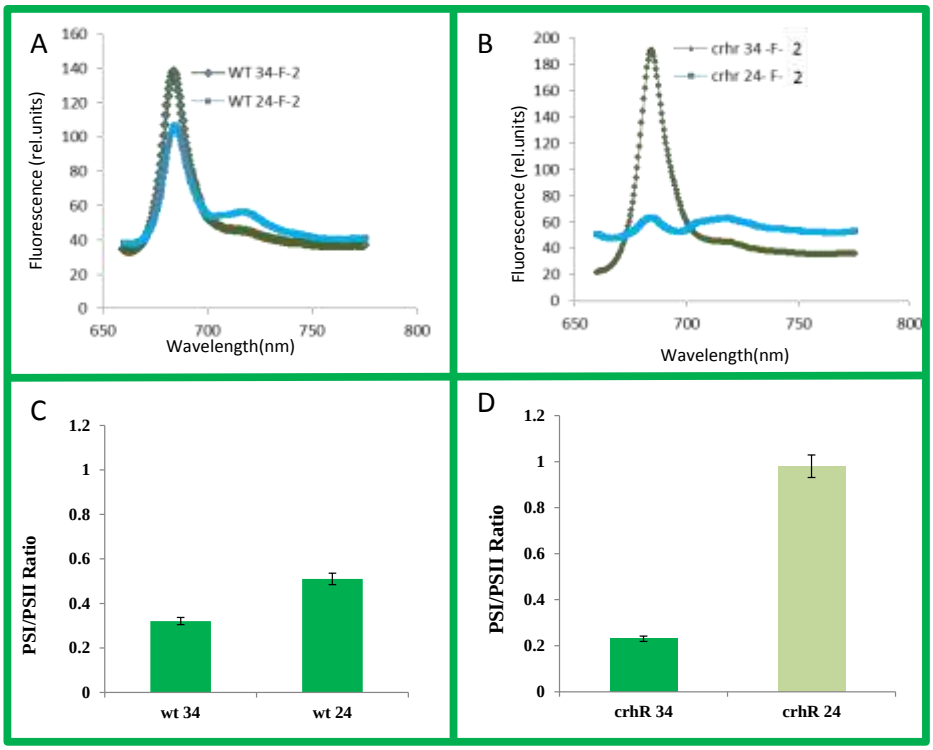


Fig.6.2. Fluorescence emission spectra at 77K of F2, from wild-type and Δ*crhR* mutant cells excited at 436 nm. Spectras represent the F2 obtained after ultracentrifugation of the solubilised thylakoids of wild-type cells grown at 34^{o}C and Δ*crhR* cells that had been grown at 34^{o}C for 16 h and then grown at 24^{o}C for 72 h(A and B). In both panels solid line represents 34^{o}C and dotted line represents cells incubated at 24^{o}C for 72 h. The relative ratio of fluorescence at 730 nm and 680 nm is represented as PSI/PSII ratio. PSI/PSII wild-type cells grown at 34^{o}C and Δ*crhR* cells that had been grown at 34^{o}C for 16 h and then incubated at 24^{o}C for 72 h (C and D). F2 were excited at 436 nm and emission spectra was measured between 600 and 780 nm.

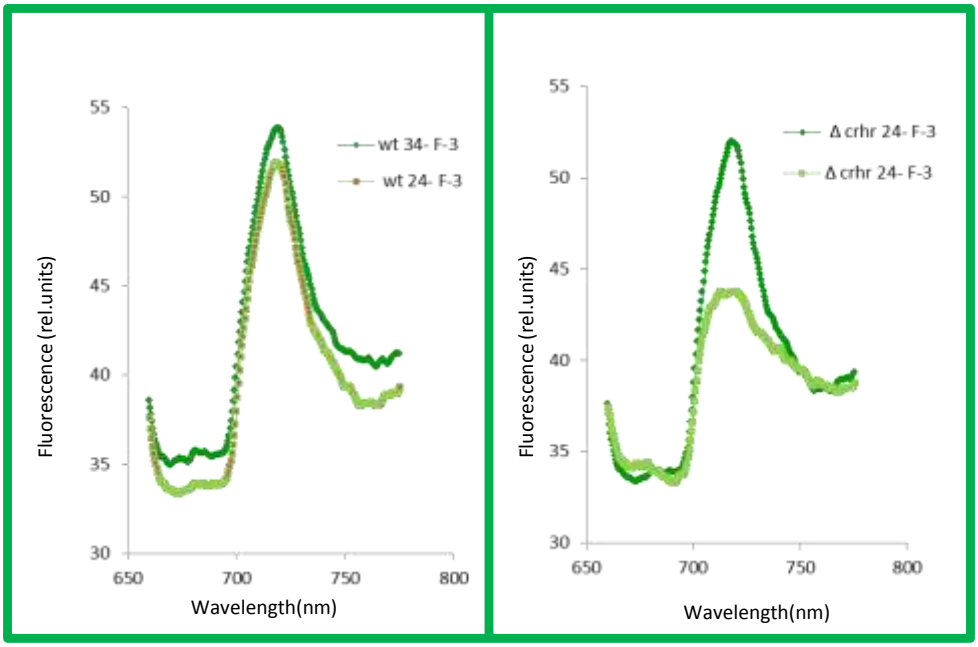


Fig.6.3. Fluorescence emission spectra at 77 K of F3, from wild-type and Δ*crhR* mutant cells after excitation at 436 nm. Spectras represent F3 of wild type cells grown at 34^{o}C and 24 ^{o}C (A) Δ*crhR* cells that had been grown at 34^{o}C for and incubated at 24^{o}C for 72 h (B).

significant decrease in the F720 peak indicating a severe loss in photosynthetic yield (Fig.6.3).

6.2.4. Changes in the levels of PSI and PSII reaction center proteins

The changes in intracellular pigment content in the wild-type and Δ*crhR* cells under low temperature reflect the changes in the levels of PSI and PSII proteins in the cells. In order to examine a possible role of *crhR* in low temperature acclimation, the changes in the relative levels of PSI and PSII reaction center proteins were analysed immunologically using antisera raised against different reaction center core proteins.

Immunodetection of reaction center proteins with antisera raised against, the PsaA/B proteins of the PSI reaction center, the D1 protein of PSII, is presented in Figure.6.4. No significant differences between the photosystem related proteins were observed between the two strains at optimum growth temperature. In contrast, the levels of PSI and PSII proteins decreased in Δ*crhR* on exposure to low temperature (Fig.6.4).

Thus, immunoblotting of PsaA and PsaB proteins under low temperature also indicated loss of PSI in the Δ*crhR* cells at low temperature indicating unaltered photosystem stoichiometry in the mutant when compared to wild-type cells at low temperature (Fig.6.4).

6.2.5. Changes in oxidoreduction of PQ pool and electron transport

The oxidoreduction of PQ pool and electron transport were characterized in detail by using PAM measurements. The inital rise in chlorophyll fluorescence transients followed by a decrease, states that the redox state of PQ pool was dynamic in wild-type and Δ*crhR* at 34^{o}C (Fig. 6.5A,B). When the cultures shifted to low temperature (24^{o}C), wild-type retained its ability to show initial rise followed by a decrease in fluorescence transient during AL illumination (Fig.6.5C), however, Δ*crhR* lost its ability to performe the same and attained a stationary phase in which the PQ pool was mostly be in oxidized state (Fig.6.5D). These results show that wild-type at 34^{o}C and 24^{o}C was able to perfom oxidoreduction of PQ pool.

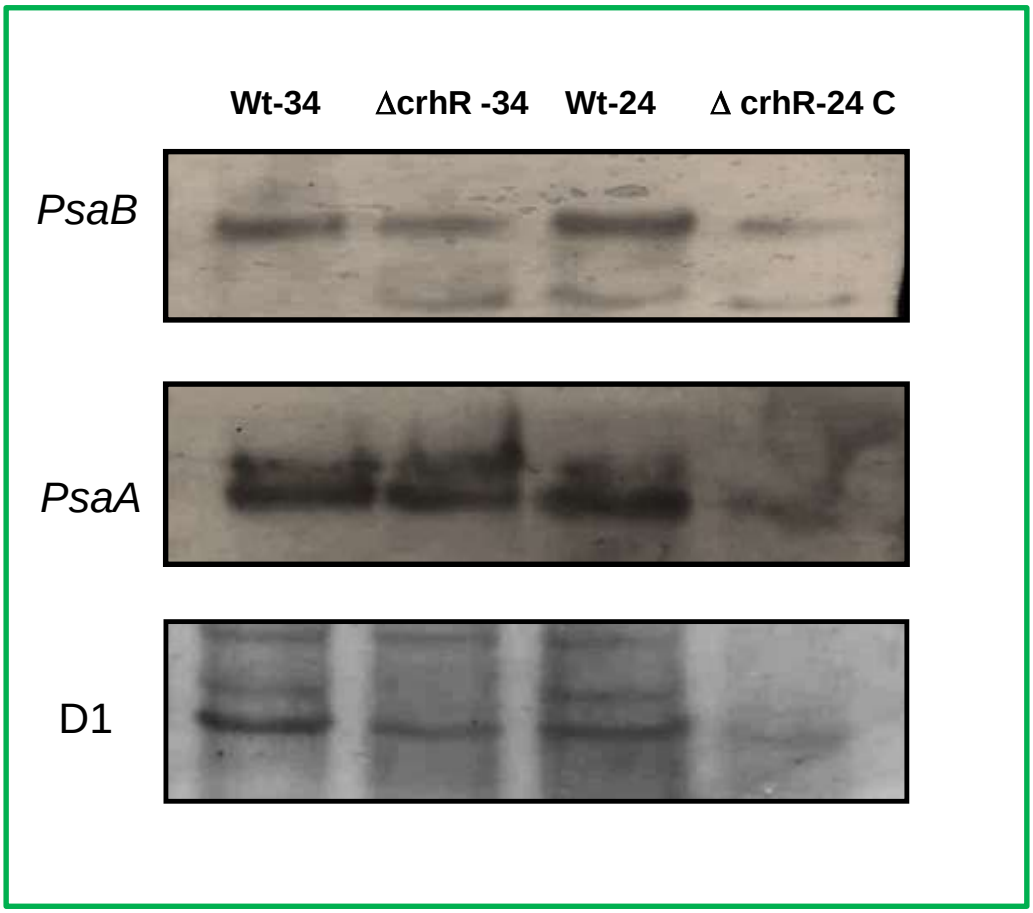


Fig.6.4. Immunoblotting analysis of thylakoid membranes isolated from wild-type and Δ*crhR* at 34^{o}C and 24^{o}C. The blots were probed with PSI specific antibodies PsaB, PsaA and PSII specific antibody, D1. Equal protein concentration was loaded on each lane.

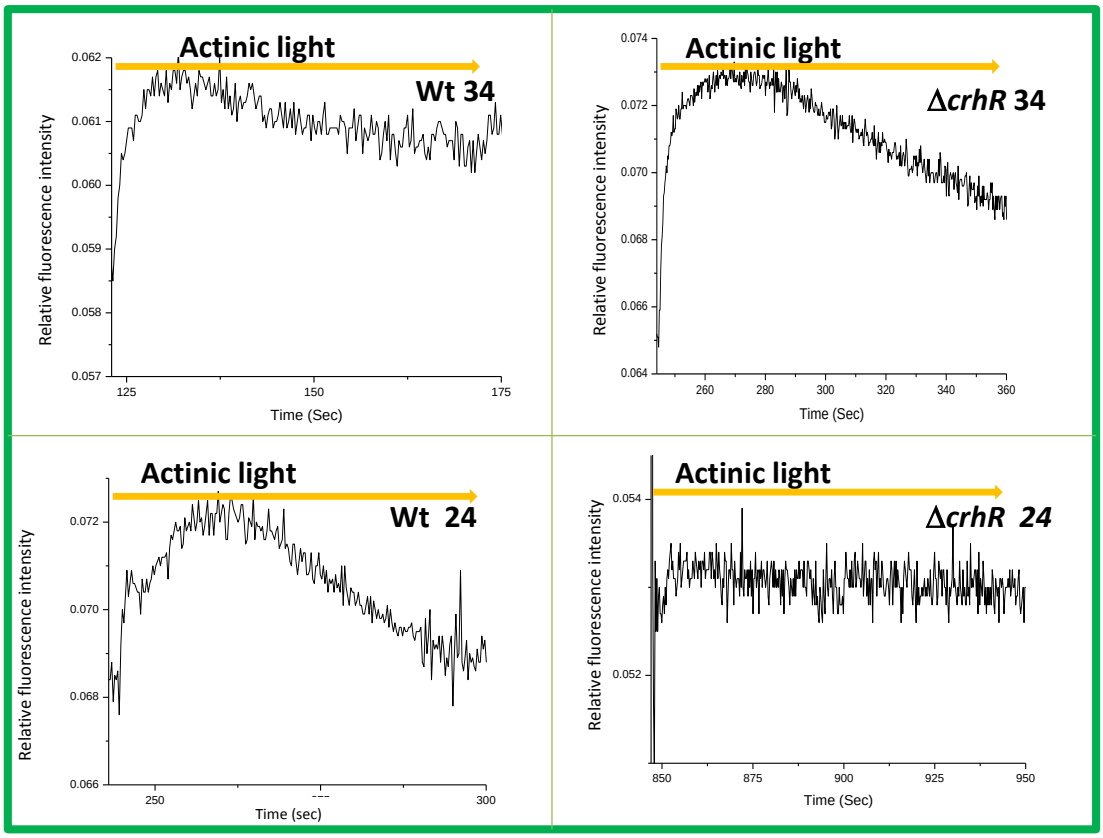


Fig.6.5. Changes in the intensity of chlorophyll fluorescence upon the application of actinic light (AL; 37 µmol photons m^{-2} s^{-1}) to monitor the redox state of PQ pool thereby to assess the functional regulation of photosystem stoichiometry in wild-type and Δ*crhR* cells at 34^{o}C (A,B) and 24^{o}C (C,D).

The dynamic nature of oxidoreduction of PQ pool can be correlated to functional regulation of photosystem stoichiometry. Thus, the results show that deletion of *crhR* gene showed defect in regulation of photosystem stiochiometry in *crhR* mutant strains.

6.2.6. Photosystem related gene expression changes

It was stated that State transitions are dependent on the changes in the redox state of the PQ pool (Mullineaux and Allen, 1990). Earlier studies revealed that the redox state of the PQ pool is involved in the transcriptional regulation of genes encoding PSI and PSII proteins (Pfannschmidt et al., 1999). These results suggested that the redox poise of the PQ pool is critical for regulation of the photosystem reaction center genes as well as state transitions. Earlier report suggests that when the cells are in oxidised state PSI genes are strongly repressed compared to the PSII genes. As the redox state of PQ has been changed due to low temperature (5^{th} chapter), in order to show that *crhR* is probably involved in early signalling process during transcriptional regulation during the process of low temperature signalling we further investigated the changes in the gene expression in the wild-type and Δ*crhR* cells at 34^{o}C and the cells incubated at 24^{o}C for 20, 60, and 180 min. Since, the PQ pool oxidation is known to repress the PSI reaction centre genes, the DNA microarray data was analysed for the extent of repression of genes coding for thylakoid membrane proteins, especially PSI and PSII proteins, by clustering the low temperature repressible genes. Cluster analysis was performed as described in materials and methods. A clustering algorithm arranges genes according to their similarity in expression profiles across all of the array experiments, such that genes with similar expression patterns are clustered together. A total of 140 low-temperature repressible genes were extracted by filtering all the down-regulated genes using Microsoft Excel work sheet and then submitted for cluster analysis. The data are graphically displayed in coloured boxes that represent the variation in transcript abundance, for each gene: shades of green represent decreases in mRNA level (down regulated genes) and red

represent the increased mRNA levels (up-regulated of genes). Saturation of the colour corresponds to the magnitude of the differences (Fig.6.6). This approach revealed three major groups of genes according to their behaviour in response to low temperature conditions as well as Δ*crhR* mutation. The first group contains genes coding for chaperonins, *groES, groEL1* and *groEL2*, which were induced during prolonged incubation of wild-type cells at 24^{o}C, but strongly repressed in the Δ*crhR* mutant (Prakash et al., 2010). In the second group, the genes that are repressed due to low temperature incubation in the wild-type and Δ*crhR* cells were represented. However, the extent of repression is much stronger in the Δ*crhR* mutant at low temperature when compared to the wild-type cells. Genes coding for *psaA, psaB, psaD, psaC, psaK, psaF, psaL of* PSI and *cpcB, cpcC2, cpcC1* and *cpcD* of phycobilisome protein belong to this group (indicated with * mark in the Fig.6.6). Genes that belong to group 3 are equally repressed both in the wild-type and Δ*crhR* mutant. The extent of repression is not as strong as the repression of the PSI genes in both wild-type and Δ*crhR* mutant at low temperature (Fig.6.6). Thus, it is clear from the experiments with PQ redox state analysis and DNA microarray analysis that lack of *CrhR* in the cells at low temperature leads to the formation of oxidized PQ pool signalling the repression of PSI genes (Allen , 1995).

6.2.7. Repression of *psaAB* genes under low temperature

Change in the repression of *psaA* and *psaB* genes under low temperature in the Δ*crhR* mutant was further examined by northern blotting analysis (Fig.6.7). As observed by the microarray analysis (Fig.6.6), the expression of the *psaAB* genes in wild-type cells was initially repressed and then recovered back to the original level during further incubation at 24^{o}C. In contrast, the *psaAB* genes in the Δ*crhR* cells were significantly repressed and could not recover back to the original level during further incubation at 24^{o}C. The level of mRNA is the result of balance between the rate of transcription and the rate of degradation. Therefore, the low

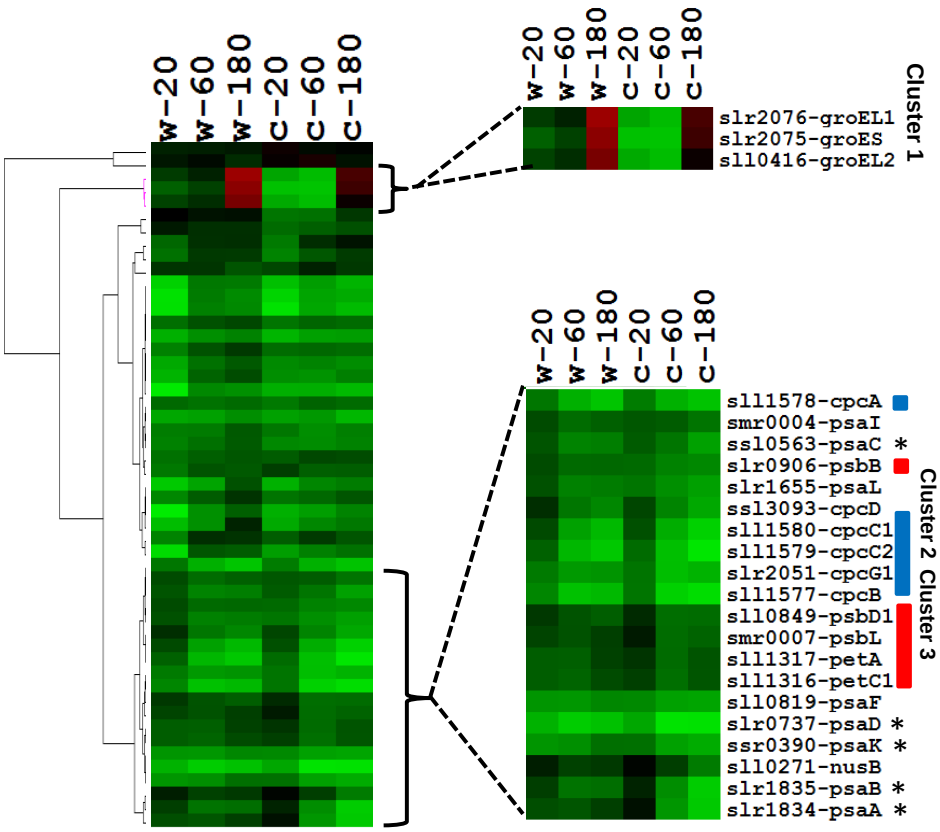


Fig.6.6. Cluster analysis of genes repressed by low temperature stress in wild-type and in Δ*crhR* mutant cells, incubated at 24^{o}C for 20, 60 and 180 min. Hierarchical clustering was performed with Cluster 3.0. The resulting clusters were visualized with Tree view 1.60 (http://rana.lbl.gov/EisenSoftware.htm). Shades of red represent increases and shades of green represent decreases in mRNA levels, relative to untreated cells, and the saturation of the color corresponds to the magnitude of the differences, whereas the black colour indicates an undetectable change in transcription levels. Cluster 1 represent genes upregulated in wild type cells after prolonged incubation, while they are strongly repressed in the mutant during initial phase of low temperature treatment and not upregulated during prolonged incubation. Cluster 2 represents genes especially genes of PBS which were strongly repressed in both wild- type and Δ*crhR* cells during low temperature incubation. Cluster 3 represents some PSII and Cytocrome b6/f complex genes with slightly down regulated or with unaltered gene expression. PSI genes, *psaA, psaB psaK, psaD* and *psaC* are indicated with * symbol. These are relatively strongly repressed in the Δ*crhR* mutant cells due to low temperature treatment.

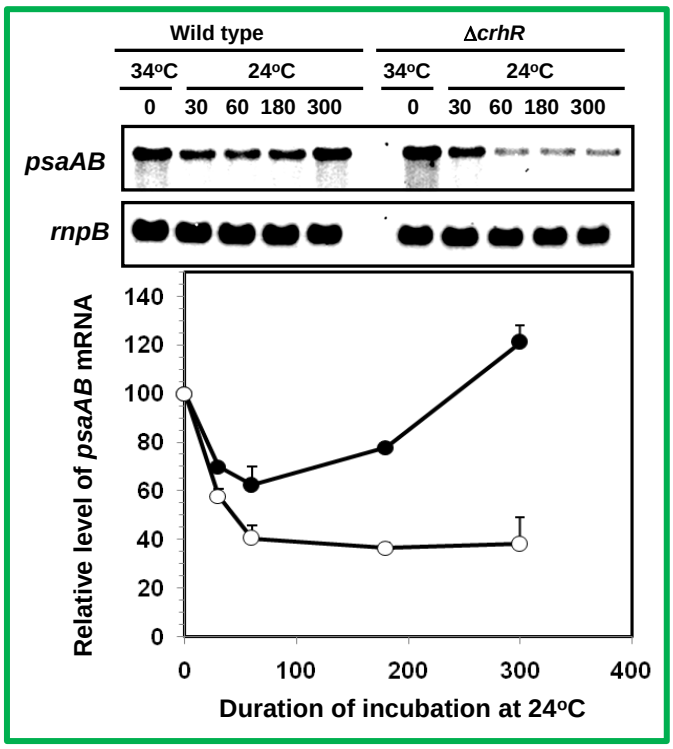


Fig.6.7. Northern blotting analysis of the expression of the *psaAB* gene in wild-type and Δ*crhR* cells. RNA was extracted from wild-type and Δ*crhR* cells that had been grown at 34 ^{o}C and then incubated at 24 ^{o}C for 30, 60, 180, 300 min. Aliquots (15 μg) of the extracted RNA were fractionated on a 1.2% agarose gel that contained 1.4 M formaldehyde. A **1646**-bp DNA fragment that included the *psaAB* genes was amplified by PCR with primers 5'TTT CCT TTA AGG ATG AAT CCA G3' and 5'TTA CATCAT GCC CAT GCC3' and used as the probe. ●, wild-type cells; ○, Δ*crhR* cells. For normalization, signals were compared with signals due to the *rnpB* gene. Similar results were obtained in two independent experiments, and the results of one of these experiments are shown here.

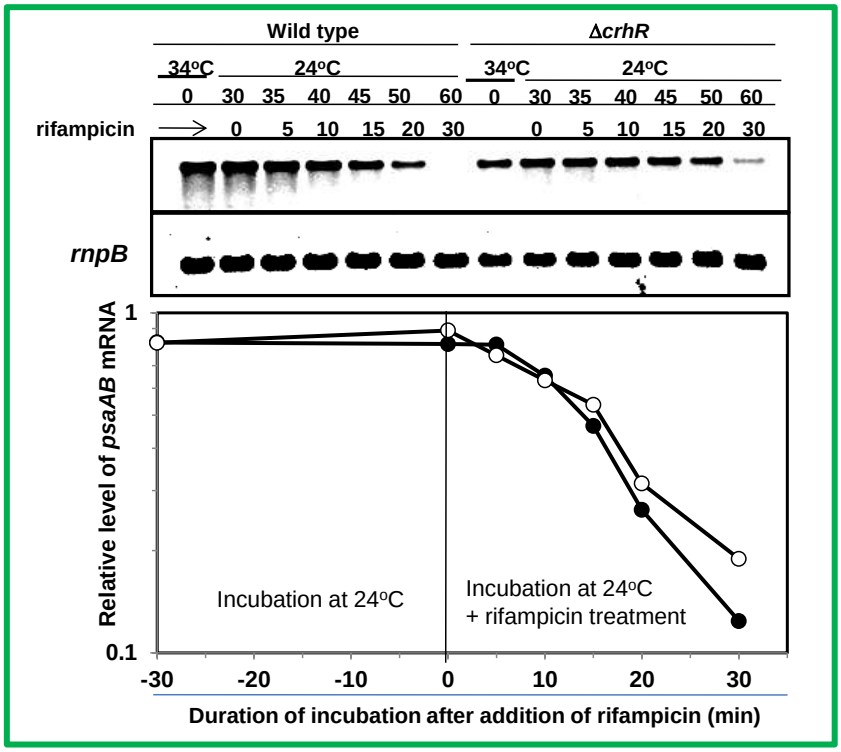


Fig.6.8. Changes in the stability of the *psaAB* transcripts due to the mutation of the *crhR* gene. Levels of *psaAB* transcripts in wild-type and Δ*crhR* cells, after cells had been grown at 34°C and then incubated at 24°C for 30 min. Rifampicin (100 μg/ml) was added to cultures of low-temperature-stressed cells and then the cells were harvested at various times for extraction of RNA. Signals detected with a luminescence image analyzer were used for calculations of the relative abundance of *psaAB* transcripts and these values are plotted on a logarithmic scale against the time after addition of rifampicin. Similar results were obtained in three independent experiments, and the data are shown as means ± S.D.

temperature induced changes in the levels of *psaAB* mRNA due to the mutation of *crhR* gene could be related to changes in the rate of transcription and /or changes in the stability of mRNA.

6.2.8. Repression of *psaAB* genes under low temperature is not due to post transcriptional control involving CrhR protein

To elucidate whether CrhR regulates the *psaAB* transcript post transcriptionally at the level of mRNA stability, or in absence of CrhR, the oxidized PQ pool signals the repression of PSI genes, we analysed the decay kinetics of *psaAB* transcripts in the presence of rifampicin, as an inhibitor of transcription. In this experiment, wild-type and Δ*crhR* mutant cells, which had been grown at 34^{o}C for 16 h, were incubated at 24^{o}C for 30 min, before the assay of decay kinetics (Fig.6.8). Figure 6.8; depict the stability of *psaAB* transcripts in wild-type and Δ*crhR* cells after incubation of the cells at 24^{o}C for 30 min. The *psaAB* transcripts were equally stable (Fig.6.8), suggesting that the repression of *psaAB* genes was not due to differential stability of the transcripts of these genes. It seems likely that CrhR might not be involved in the post transcriptional regulation of *psaAB* gene expression. Thus, it is inferred from the PQ redox state analysis, steady state of *psaAB* mRNA levels and mRNA stability analysis that the strong repression of *psaAB* in absence of *crhR* is due to the oxidized PQ signalling, but not due to post transcriptional control. Importantly, Δ*crhR* cells failed to balance the function of photosystems at low temperature and failed to acclimatize to low temperature.

6.3. Conclusion

Our data provide significant insights to the adaptation of *Synechocystis* to low temperature acclimation. We show that adjustment of cellular processes (e.g.state transitions and adjustment of photosystem stoichiometry) are necessary to reverse the effects of excitation imbalance generated due to low temperature treatment. A significant finding relates to the

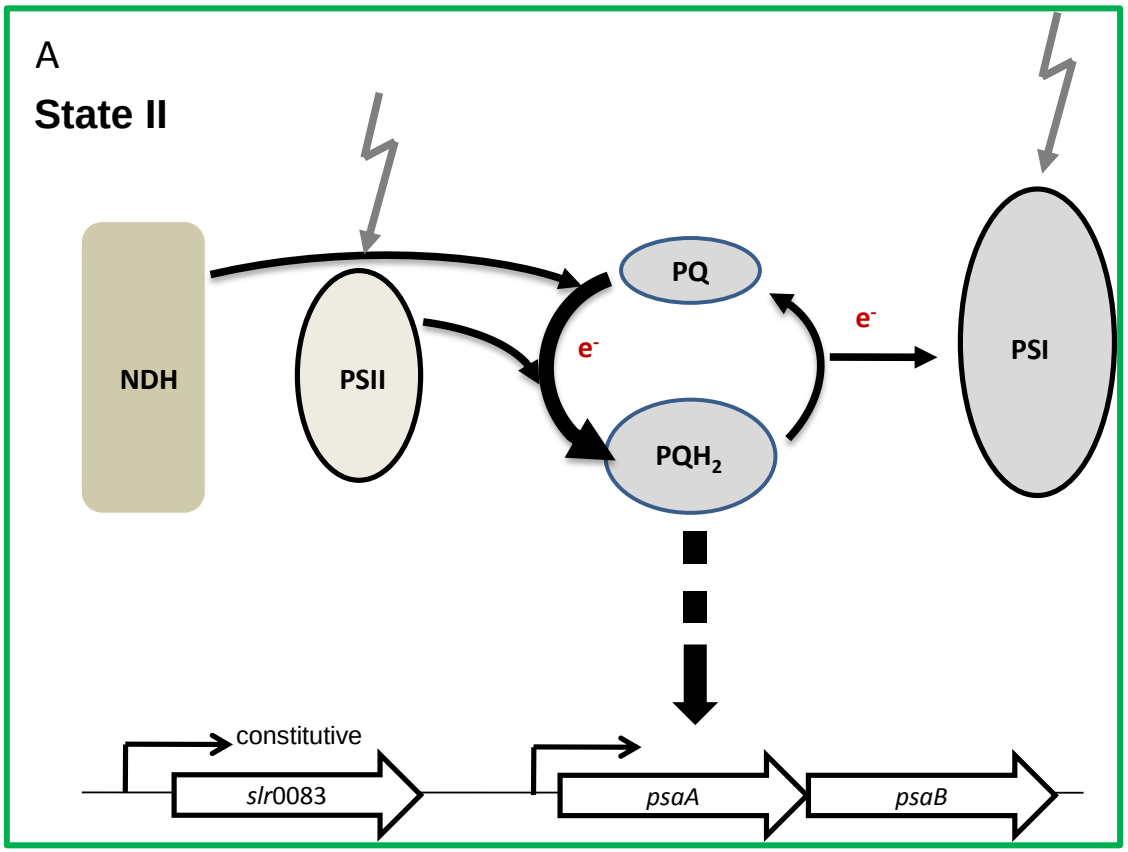
growth of *Synechocystis* under low temperature. Our results have led to the identification of necessary cellular adaptations that could enable the growth of *Synechocystis* during low temperature adaptation in wild type-cells is due to the expression of *crhR*.

The PSI genes were repressed in the wild-type cells and recovered back to original levels during incubation at low temperature. However, PSI genes were repressed strongly and were not recovered back to original level in the Δ*crhR* mutant. *psaAB* mRNA is equally stable in the wild-type and Δ*crhR* mutant cells during low temperature, suggesting *CrhR* is not directly involved in post transcriptional regulation of *psaAB* expression. Oxidized PQ pool might be the signal for repression of *psaAB* genes in the mutant. Alteration in the photosystem stoichiometry is probably a mechanism of low temperature acclimation. The *crhR* mutant cells failed to regulate photosystem stoichiometry at low temperature.

Taken together these observations indicate that the ability to respond to environmental changes by sensing shifts in redox potential has been conserved between prokaryotic and eukaryotic photosynthetic organisms and that cold regulated RNA helicase is involved in the regulation of genes expressed in response to these changes. This conservation supports the proposal that the physiological requirement for the rapid regulation of gene expression by redox signaling is a factor leading to the maintenance of chloroplast and mitochondrial genetic systems (Allen, 1993). Although the plastoquinol- redox control model proposed by Allen (1995) emphasizes the regulation of photosynthetic gene expression, the data presented here reveal a similar pattern of RNA helicase expression regulated by redox status. Although an RNA helicase would not be involved directly in the light-harvesting reactions per se, coordinate regulation of *crhR* expression implies that modulation of RNA secondary structure is required during conditions that elicit electron flow. CrhR-induced RNA unwinding activity could probably remove secondary structures that inhibit the efficient translation of mRNAs whose products are required under these conditions.

6.4. Message

Functional balancing between photosystems with respect to light regime has been well documented (Keren and Ohad, 1998). Under changing light intensities, energy redistribution between the two photosystems occurs. It has been reported that the involvement of mobility of phosphorylated LHCII in plants and mobility of PBS in cyanobacteria leads to energy redistribution between the photosystems for functional optimization and coordination of either photosystems (Tikkanen et al., 2010; Mullineaux et al., 1997). Optimization of photosystem stoichiometry by changing oligomerization of PSII and PSI complexes and regulated synthesis of genes coding for either photosystems occurs when there is a functional imbalance between photosystems (Murakami and Fujita, 1991; Pfannschmidt et al., 1999). Controlled expression of genes by redox signalling, making sure of altered ratio between PSI and PSII for functional balancing under changed light conditions has been published in the literature (Allen, 1992, 1995; Keren and Ohad, 1998). However, to the best of our knowledge, most of these studies were focussed with respect to adaptation of plants or cyanobacteria to changing light intensities. Here, we studied the low temperature induced energy redistribution between photosystems as an acclimative response. In addition such balancing requires essentially an RNA helicase, *CrhR,* in the cyanobacterium *Synechocystis.* Cyanobacteria are generally under state 2 under low light and dark conditions (Mullineaux and Allen, 1990; Falk et al., 1995; Campbell et al., 1998). As a result, the PQ is under reduced state leading to constitutive expression of gene coding for RNA helicase and genes for PSI (Fig.6.9A). *Synechocystis* cells transiently enter into state 1 from state 2 and back to state 2, when shifted from 34^{o}C to 24^{o}C, as evidenced from transient down-regulation of *psaAB* gene during early phase of low temperature incubation (Fig.6.9). Enhancement in PQ pool reduction could be a result of more supply of electrons from respiratory reductants and also could be due to enhanced rate of PSII activity occur under prolonged incubation of cells


A
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slr0083
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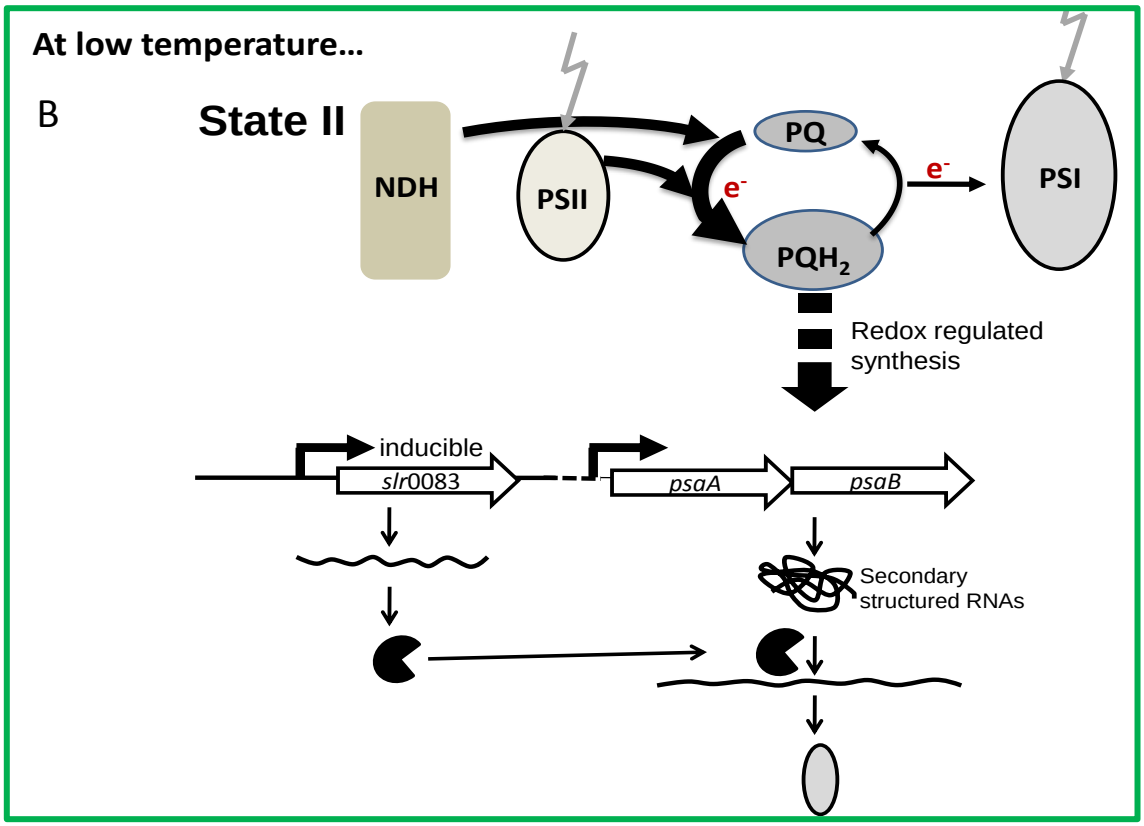
At low temperature...
B
State II
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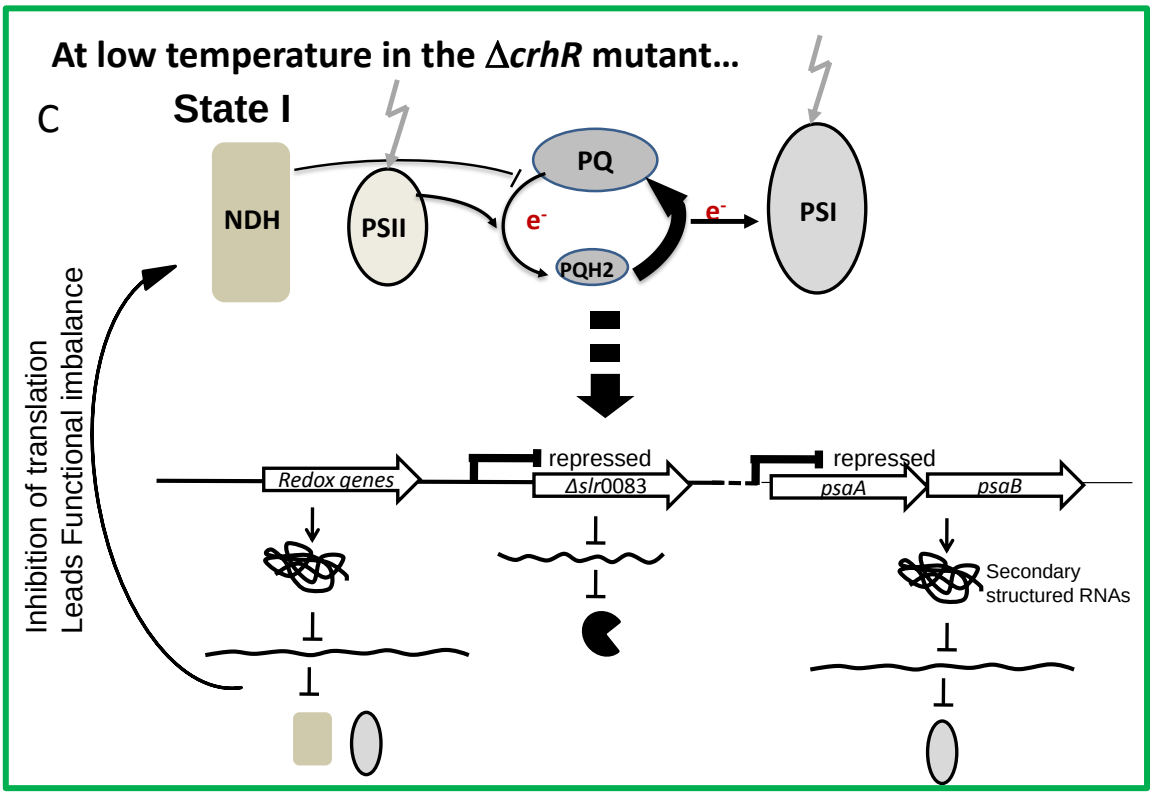

6.9. A scheme for control of RNA helicase in control of redox state of PQ pool and regulation of photosystem stoichiometry for optimal photosynthesis at low temperature. (A). Balanced functions of photosystems at 34^{o}C in the wild-type. PQ pool is kept under reduced state due to electron flow from the respiratory reductants and PSII. The cells are in state 2. RNA helicase gene is constitutively expressed. Broken arrow indicates path of redox signal transduction for controlled expression of PSI genes. (B) Low temperature induced changes in state transitions in the wild-type. Low temperature induced enhanced electron flow from respiratory NDH and from PSII to PQ pool, resulting in strong redox signal to induced transcription of *crhR* gene. Dashed lines between the open reading frames indicates different locations of the genes on the genome of *Synechocystis*. (C) Δ*crhR* cells failed to exhibit low temperature induced state transitions. Δ*crhR* cells are locked in state 1. PQ pool is in oxidized state as a result of inhibition of electron flow from respiration and imbalance between photosystem function. This imbalance is a result of lack of *crhR* which is supposed to resume low temperature affected translation of redox regulated genes. As PQ pool is in oxidized state the PSI genes were kept strongly repressed. Arrows indicate electron transfer reactions and gene expression levels, and thunderbolts designate light that sets into motion the redox reactions in photosystems. The thickness of each arrow is an approximate indication of the rate of the corresponding reactions.

coding for RNA helicase, *crhR* was disrupted. Thus we suggest from our results that CrhR is essentially required for photosysthetic acclimation under low temperature.

Summary

7. Summary

- The expression of *slr0083* gene and corresponding product (CrhR protein) is regulated by stress conditions. The mRNA, protein amount was increased only when cells were shifted to sub optimal temperature conditions.
- Inactivation of the *slr0083* mutant in *Synechocystis* was done by targeted deletional inactivation of *crhR* gene. All the wild-type copies of the *crhR* gene were in the chromosome were replaced with the spectinomycin gene cassette resulting in a viable photoautotrophic mutant strain Δ*slr0083* (or Δ*crhR*) with similar phenotypical features of the wild-type when grown under optimum growth temperatures. The analysis of pigment content of cells grown under optimum conditions showed that the mutant contained equal amount of PC, chlorophyll *a*.
- Acclimation of cyanobacterial cells to low temperature stress condition led to phenotypical modifications of mutant Δ*crhR*. Δ*crhR* cells exhibited slow growth and a bleached phenotype under low temperature. The cells of Δ*crhR* remained viable even after low temperature treatment. Δ*crhR* cells appeared as cells with bi-lobed nucleoids at low temperature. This is consistent with observed slow growth phenotype (delayed rate of cell division) of the Δ*crhR*. Alteration in the cell size and thylakoid membrane structure was observed due to low temperature. The thylakoid membranes were found to be distorted in the wild-type.
- Detailed functional analysis of cells Δ*crhR* was performed during acclimation to low temperature. Loss of chlorophyll was observed in the Δ*crhR* cells at low temperature. Comparative analysis of pigment concentrations showed that pigment degradation occurred more quickly in the mutant strain under low temperature incubation. These modifications were accompanied by differences in the cell absorption spectra which reflect the pigment content of cyanobacterial cells.

- Absorption spectra indicated a preferential loss of chlorophyll over PC in the mutant upon low temperature treatment. Low temperature leads to an alteration in PC/chl ratio in wild-type and Δ*crhR* cells. A blue shift in the red region of absorption spectrum was observed in the wild-type cells upon low temperature treatment. However such shift was not seen in the mutant.
- Uncoupling of PBS from PSII in the wild-type cells was observed upon low temperature. Such uncoupling was not seen in the mutant. An increase in the PSI monomer content and PSII dimer content upon low temperature was observed in the wild-type. However increase in PSII dimer content was not seen in the mutant. All the above results indicate that the wild-type cells adopt different mechanisms to cope up the low temperature stress while the mutant was unable to exhibit these adaptive responses.
- Δ*crhR* cells failed to operate short term acclimative response of energy redistribution at low temperature. Wild-type cyanobacterial cells remained in state 2 with reduced PQ pool, while Δ*crhR* mutant cells are in state 1 with oxidzed PQ pool at low temperatures.
- The PSI genes were repressed initially in the wild-type cells and recovered back to original levels during prolonged incubation at low temperature. However PSI genes were repressed strongly and were not recovered back to original level in the Δ*crhR* mutant.
- *psaAB* mRNA is equally stable in the wild-type and Δ*crhR* mutant cells during low temperature, suggesting *crhR* is not directly involved in post transcriptional regulation of *psaAB* expression. Oxidized PQ pool might be the signal for repression of *psaAB* genes in the mutant. The *crhR* accumulation occurs *in vivo* upon exposure to low temperature stress and RNA helicase was not found to be involved in the signalling

and under low temperature. It was concluded that PQ orchestrates the processes involved in the regulation of photosystem stoichiometry of photosystems associated genes of PBS and RNA helicase could be involved in unwinding of the secondary structured mRNAs.

- Using mutant that lack RNA Helicase protein in the cell which affected low temperature acclimation, it was demonstrated that the RNA Helicase *crhR* correspond to the photosystem functional co-ordination during low temperature acclimation. Alteration in the photosystem stoichiometry is probably a mechanism of low temperature acclimation. The *crhR* mutant cells were failed to regulate photosystem stoichiometry at low temperature.

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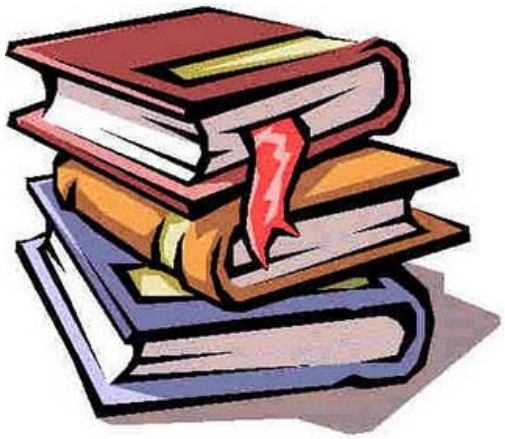
Literature cited



References

Adamcik, J., Viglasky, V., Valle, F., Antalik, M., Podhradsky, D.and Dietler, G. (2002) Effect of bacteria growth temperature on the distribution of supercoiled DNA and its thermal stability. Electrophoresis 23: 3300-3309.

Adamska, I., Roobol-Boza, M., Lindahl, M. and Andersson, B. (1999) Isolation of pigment- binding early light-inducible proteins from pea. Eur J. Biochem. 260: 453-60.

Aguilar, P.S., Hernandez-Arriaga, A.M., Cybulski, L.E., Erazo, A.C. and De Mendoza (2001) Molecular basis of thermosensing: A two-component signal transduction thermometer in *Bacillus subtilis*. EMBO J. 20: 1681-1691.

Aizawa, K. and Fujita, Y. (1992) Regulation of PS I formation induced by light quality observed with *Synechocystis* PCC6714. In: Murata, N. (ed) Research in Photosynthesis, Vol IV, Kluwer Academic Publishers, Dordrecht, the Netherlands pp 329–332.

Aizawa,K. and Fujita, Y. (1997) Regulation of synthesis of PSI in the cyanophytes *Synechocystis* PCC6714 and Plectonema boryanum during the acclimationof the photosystem stoichiometry to the light quality. Plant Cell Physiol. 38: 319–326.

Alfonso, M., Perewoska, I. and Kirilovsky, D. (2000) Redox control of psbAgene expression in the cyanobacterium *Synechocystis* PCC 6803.Involvement of the cytochrome b_6/f complex. Plant Physiol. 122: 505–515.

Allen, J.F. (1992) Protein phosphorylation in regulation of photosynthesis. Biochim Biophys Acta 1098: 275–335.

Allen, J.F. (1992) Protein phosphorylation in regulation of photosynthesis. Biochim. Biophys. Acta1098: 275–335.

Allen, J.F. (1993) Redox control of gene-expression and the function of chloroplast genomes – an hypothesis. Photosynth. Res. 36: 95–102.

Allen, J.F. (1995) Thylakoid protein phosphorylation, state 1-state 2 transitions, and photosystem stoichiometry adjustment: redox control at multiple levels of gene expression. Physiol. Plant 93: 196–205.

Allen, J.F. (2003a) Botany. State transitions – a question of balance. Science 299: 1530–1532.

Allen, J.F. and Forsberg, J. (2001) Molecular recognition in thylakoid structure and function. Trends. Plant Sci. 6: 317-326.

Allen, J.F. and Pfannschmidt, T. (2000) Balancing the two photosystems: photosynthetic electron transfer governs transcription of reaction centre genes in chloroplasts. Phil. Trans. R. Soc. Lond. B Biol. Sci. 355: 1351–1359.

Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. Nature 291: 25-29.

Allen, J.F., Sanders, C.E. and Holmes, N.G. (1985) Correlation of membrane protein phosphorylation with excitation energy distribution in the cyanobacterium *Synechococcus* PCC 6301. FEBS Lett. 193: 271-275.

Allen, M.M. and Smith, A.J. (1969) Nitrogen chlorosis in blue-green algae. Arch. Microbiol. 69:114-120.

Anderson, JM., Chow, W.S. and Park, Y.I. (1995) The grand design of photosynthesis: acclimation of the photosynthetic apparatus to environmental cues. Photosynth. Res. 46: 129–139.

Andersson, B. and Barber, J. (1996). Mechanisms of photodamage and protein degradation during photoinhibition of photosystem II. In: Baker NR (ed.) Photosynthesis and the Environment, pp. 101–121. Dordrecht: Kluwer Academic Publishers.

Aoyama, T. and Takanami, M. (1988) Supercoiling response of *E. coli* promoters with different spacer lengths. Biochim.Biophys.Acta 949: 311-317.

Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y. and Wad, K. (1974) Photochemical activity and components of membrane preparations from bluegreen algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. Biochim Biophys Acta 357:231.245.

Asada, K., Heber, U. and Schreiber, U. (1993) Electron flow to the intersystem chain from stromal components and cyclic electron flow in maize chloroplasts, as determined in intact leaves by monitoring redox change of P700 and chlorophyll fluorescence. Plant Cell Physiol. 34: 39–50.

Aubourg, S., Kreis, M. and Lecharny, A. (1999) The DEAD box RNA helicase family in *Arabidopsis thaliana*. Nucleic Acids Res. 27: 628–636.

Bailey, S., Silva, P., Nixon, P., Mullineaux, C., Robinson, C. and Mann, N. (2001) Auxiliary functions in photosynthesis: the role of the FtsH peptidase. Biochem. Soc. Trans. 29: 455.

Bailey, S., Thompson, E., Nixon, P.J., Horton, P., Mullineaux, C.W., Robinson, C. and Mann, N.H. (2002) A critical role for the Var2 FtsH homologue of *Arabidopsis thaliana* in the photosystem II repair cycle in vivo. J. Biol. Chem. 277: 2006-2011.

Barber, J. (2002) Photosystem II: a multisubunit membrane protein that oxidises water. Curr. Opin. Struct. Biol. 12: 523-530.

Bartsevich, V.V. and Pakrasi, H.B. (1997) Molecular identification of a novel protein that regulates biogenesis of photosystem I, a membrane protein complex. J. Biol. Chem. 272: 6382–6387.

Bartsevich, V.V. and Shestakov, S.V. (1995) The *dspA* gene product of the cyanobacterium *Synechocystis* sp.strain PCC 6803 influences sensitivity to chemically different growth inhibitors and has amino acid similarity to histidine protein kinases. Microbiology 141: 2915-2920.

Beckering, C.L., Steil, L., Weber, M.H., Volker, U. and Marahiel, M.A. (2002) Genomewide transcriptional analysis of the cold shock response in *Bacillus subtilis.* J Bacteriol. 184: 6395–6402.

Bellafiore, S., Barneche, F., Peltier, G. and Rochaix, J.D. (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892–895.

Bendall, D.S. and Manasse, R.S. (1995). Cyclic photophosphorylation and electron transport. Biochim. Biophys. Acta 1229: 23–38.

Ben-Shem, A., Frolow, F. and Nelson, N. (2003) Crystal structure of plant photosystem I. Nature 426: 630-635.

Berry, J. and Bjorkman, O. (1980) Photosynthetic response and adaptation to temperature in higher plants. Annu. Rev. Plant Physiol. 31: 491-543.

Bizebard, T., Ferlenghi, I., Iost, I. and Dreyfus, M. (2004) Studies on three *E. coli* DEAD-box helicases point to an unwinding mechanism different from that of model DNA helicases. Biochemistry 43: 7857–7866.

Bonaventura, C. and Myers, J. (1969) Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*. Biochim. Biophys. Acta189: 366-383.

Bond, A.T., Mangus, D.A., He, F. and Jacobson, A. (2001) Absence of Dbp2p alters both nonsense-mediated mRNA decay and rRNA processing. Mol. Cell Biol. 21: 7366–7379.

Boonyaratanakornkit, B.B., Simpson, A.J., Whitehead, T.A., Fraser, C.M. and El-Sayed, N.M.A. and Clark, D.S. (2005) Transcriptional profiling of the hyperthermophilic methanarchaeon *Methanococcus jannaschii* in response to lethal heat and non-lethal cold shock. Environ. Microbiol. 7: 789–797.

Brander, K.A., Mandel, T., Owttrim, G.W. and Kuhlemeier, C. (1995) Highly conserved genes coding for eukaryotic translation initiation factor eIF-4A of tobacco have specific alterations in functional motifs. Biochim.Biophys. Acta 1261: 442–444.

Bryant, D.A. (1994) The Molecular Biology of Cyanobacteria. Dordrecht :Kluwer Academic Publishers.

Campbell, D. (1998) Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiol Mol. Biol. Rev. 62: 667-683.

Carlberg, I., Rintamäki, E., Aro, E.M. and Andersson, B. (1999) Thylakoid protein phosphorylation and the thiol redox state. Biochemistry 38: 3197-3204.

Carty, S.M., Sreekumar, K.R. and Raetz, C.R.H. (1999) Effect of cold shock on lipid a biosynthesis in *Escherichia coli*: Induction at 12°C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. J. Biol. Chem. 274: 9677-9685.

Chamot, D. and Owttrim, G.W. (2000) Regulation of cold shock induced RNA helicase gene expression in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. 182: 1251–1256.

Chamot, D., Colvin, K.R., Kujat-Choy, S.L. and Owttrim, G.W. (2005) RNA structural rearrangement via unwinding and annealing by the cyanobacterial RNA helicase, CrhR. J Biol. Chem. 280: 2036–2044.

Chamot, D., Magee, W.C., Yu, E. and Owttrim, G,W. (1999) A cold shock-induced cyanobacterial RNA helicase. J. Bacteriol. 181: 1728-1732.

Charollais, J., Drefus, M. and Iost, I. (2004) CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. Nucleic Acids Res. 32: 2751–2759.

Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M. and Iost, I. (2003) The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*. Mol. Microbiol. 48: 1253–1265.

Chen, Y. Z., Zhuang, W. and Prohofsky, E. W. (1992) Energy flow considerations and thermal fluctuational opening of DNA base pairs at a replicating fork: unwinding consistent with observed replication rates. J. Biomol. Struct. Dyn. 10: 415–427.

Cheung, K.J., Badarinarayana, V., Selinger, D.W., Janse, D. and Church, G.M. (2003) A microarray-based antibiotic screen identifies a regulatory role for supercoiling in the osmotic stress response of *Escherichia coli*. Genome Res. 13: 206-215.

Chitnis, V.P. and Chitnis, P.R. (1993) PsaL subunit is required for the formation of photosystem I trimers in the cyanobacterium *Synechocystis* sp. PCC 6803. FEBS Lett. 336: 330-334.

Chow, W.S., Melis, A. and Anderson, J.M. (1990) Adjustment of photosystem stoichiometry in chloroplasts improves the quantum efficiency of photosynthesis. Proc. Natl. Acad. Sci. USA 87: 7502–7506.

Chuang, R.Y., Weaver, P.L., Liu, Z. and Chang, T.H. (1997) Requirement of the DEAD-box protein Ded1p for messenger RNA translation. Science 275: 1468–1471.

de la Cruz, J., Iost, I., Kressler, D. and Linder, P. (1997) The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 94: 5201–5206.

de Marsac, N.T. and Cohen-bazire,G.(1977) Molecular composition of cyanobacterial phycobilisomes. Proc. Natl. Acad. Sci. USA 74: 1635-1639

Delepelaire, P. and Chua, N. (1979) Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 4 C: characterizations of two additional chlorophyll a-protein complexes. Proc. Nat. Acad. Sci. USA 76: 111-115.

Dimroth, P., Kaim, G. and Matthey, U. (2000) Crucial role of the membrane potential for ATP synthesis by F_1F_0 ATP synthases. J. Exp. Biol. 1: 51-59.

Diner, B.A. and Rappaport, F. (2002) Structure, dynamics, and energetics of the primary photochemistry of photosystem II of oxygenic photosynthesis. Annu. Rev. Plant Biol. 53: 551–580.

Dorman, C.J. (2006) DNA supercoiling and bacterial gene expression. Sci. Prog. 89: 151-166.

Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA 95: 14863–14868.

El-Fahmawi, B. and Owttrim, G.W. (2003) Polar-biased localization of the cold stress-induced RNA helicase, CrhC, in the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Microbiol. 50: 1439–1448.

Emlyn-Jones, D., Ashby, M. and Mullineaux, C.W. (1999) A gene required for the regulation of photosynthetic light harvesting in the cyanobacterium *Synechocystis* 6803. Mol. Microbiol. 33: 1050-1058.

Evans, E.H. and Brown, R.G. (1994) An appraisal of photosyntheticfluorescence kinetics as a probe of plant function. J. Photochem. Photobiol. B 22: 95–104.

Falk, S., Samson, G., Bruce, D., Huner, N.P.A. and Laudenbach, D.E. (1995) Functional analysis of the iron-stress induced CP43'polypeptide of PS II in the cyanobacterium *Synechococcus* sp. PCC 7942. Photosynth Res. 45: 51–60.

Fang, L., Jiang, W., Bae, W. and Inouye, M. (1997) Promoter independent cold-shock induction of cspA and its derepression at 37^{o}C by mRNA stabilization. Mol. Microbiol. 23: 355–364

Ferrer, M., Chernikova, T.N., Yakimov, M.M., Golyshin, P.N., and Timmis, K.N. (2003) Chaperonins govern growth of Escherichia coli at low temperatures. Nat. Biotechnol. 21: 266.

Fork, D.C. and Satoh, K. (1983) State I-State II transitions in the thermophilicblue-green alga (cyanobacterium) *Synechococcus lividus*. Photochem. Photobiol.37: 421–427.

Franco, R.J. and Drlica, K. (1989) Gyrase inhibitors can increase *gyrA*expression and DNA supercoiling. J. Bacteriol. 171: 6573-6579.

Frasch, W.D. (1994) The F-type ATPase in cyanobacteria: pivotal point in the evolution of a universal enzyme. In Book: The Molecular Biology of Cyanobacteria. Bryant DA. pp361-380.

Fromme, P., Jordan, P.and Krauss, N. (2001) Structure of photosystem I. BiochimBiophys Acta 1507: 5–31.

Fuhrmann, E., Gathmann, S., Rupprecht, E., Golecki, J. and Schneider, D. (2009) Thylakoid membrane reduction affects the photosystem stoichiometry in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Physiol. 149: 735–744.

Fujita, Y. (1997) A study on the dynamic features of photosystem stoichiometry: accomplishments and problems for future studies. Photosynth. Res. 53: 83–93.

Fujita, Y. and Murakami, A. (1987) Regulation of electron transport composition in cyanobacterial photosynthetic system: stoichiometry among photosystem I and II complexes and their light-harvesting antennae and cytochrome b_6/f complex. Plant Cell Physiol. 28: 1547–1553.

Fujita, Y., Murakami, A. and Ohki, K. (1987) Regulation of photosystem composition in the cyanobacterial photosynthetic system: the regulation occurs in response to the redox state of the electron pool located between the two photosystems. Plant Cell Physiol. 28: 283–292.

Fujita, Y., Murakami, A. and Ohki, K. (1990) Regulation of the stoichiometry of thylakoid components in the photosynthetic system of cyanophytes: model experiments showing that control of the synthesis or supply of chl *a* can change the stoichiometric relationship between the two photosystems. Plant Cell Physiol. 31: 145–153.

Fujita, Y., Murakami, A., Ohki, K. and Hagiwara, N. (1988) Regulation of photosystem composition in cyanobacterial photosynthetic system: evidence indicating that photosystem I formation is controlled in response to the electron transport state. Plant Cell Physiol. 29: 557–564.

Fujita, Y., Ohki, K. and Murakami, A. (1985) Chromatic regulation of photosystem in the photosynthetic system of red and blue-green algae. Plant Cell Physiol. 26: 1541–1548.

Fujita,Y., Murakami, A., Aizawa, K. and Ohki, K. (1994) Short-term and long-term adaptation of the photosynthetic apparatus: homeostatic properties of thylakoids.The Molecular Biology of Cyanobacteria.ed BryantDA (Kluwer Academic Publishers, Dordrecht, The Netherlands) 677–692.

Fuller-Pace, F.V (1994) RNA helicases: Modulators of RNA structure. Trends Cell Biol. 4: 271-274.

Funk, C. and Vermaas, W. (1999) A cyanobacterial gene family coding for single-helix proteins resembling part of the light-harvesting proteins from higher plants. Biochemistry 38: 9397-9404.

Gellert, M., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1976) Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA 73: 4474-4478.

Glazer, A.N. (1985) Light harvesting by phycobilisomes. Annu. Rev. Biophys. Biophys. Chem. 14: 47-77.

Glazer, A.N. (1989) Light guides.Directional energy transfer in a photosynthetic antenna. J. Biol. Chem. 264: 1-4.

Glick, R.E., McCauley, S.W., Grussem, W. and Melis, A. (1986) Light quality regulates expression of chloroplast genes and assembly of photosynthetic membrane complexes. Proc. Natl. Acad. Sci. USA 83: 4287–4291.

Golbeck, J.H. (1994).Photosystem I in cyanobacteria. In Book *The Molecular Biology of Cyanobacteria*. Edited by Bryant DA.pp 319-360.

Goldenberg, D., Azar, I. and Oppenheim, A.B. (1996) Differential mRNA stability of the cspA gene in the cold-shock response of *Escherichia coli*. Mol. Microbiol. 2: 241-248.

Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B. and Zhu, J.K. (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. Proc. Natl. Acad. Sci. USA 99: 11507–11512.

Goverde, R.L.J., Veld, J.H.J., Kusters, J.G. and Mooi, F.R. (1998) The psychrotrophic bacterium Yersinia enterocolitica requires expression of pnp, the gene for polynucleotide phosphorylase, for growth at low temperature (5 ^{o}C). Mol. Microbiol. 28: 555-569.

Graumann, P., Schroder, K., Schmid, R. and Marahiel, M.A. (1996) Cold shock stress-induced proteins in *Bacillus subtilis*. J.Bacteriol. 178: 4611-4619.

Graumann, P.L. and Marahiel, M.A. (1998). A superfamily of proteins that contain the cold-shock domain. Trends. Biochem. Sci. 23: 286-290.

Grossman, A.R. Schaefer, M.R., Chiang, G.G. and Collier, J.L. (1993) The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiol. Rev. 57: 725-749.

Gualerzi, C.O., Giuliodori, A.M. and Pon, C.L. (2003) Transcriptional and post-transcriptional control of cold-shock genes. J Mol. Biol. 331: 527–539.

Haldrup, A., Jensen, P.E., Lunde, C. and Scheller, H.V. (2001) Balance of power: a view of the mechanism of photosynthetic state transitions. Trends Plant Sci .6: 301-305.

Haselkorn, R. (1991) Genetic systems in cyanobacteria. Methods Enzymol. 204: 418–430.

Havaux, M., Guedeney, G., He, Q. and Grossman A,R. (2003) Elimination of high-light-inducible polypeptides related to eukaryotic chlorophyll a/b-binding proteins results in aberrant photoacclimation in Synechocystis PCC6803. Biochim. Biophys. Acta 1557: 21-33.

He, Q., Dolganov, N., Bjorkman, O. and Grossman, A.R. (2001)The high light-inducible polypeptides in *Synechocystis*PCC6803. Expression and function in high light. J. Biol. Chem. 276: 306-314.

Hebraud, M., and Potier, P. (1999) Cold shock response and low temperature adaptation in psychrotrophic bacteria. J. Mol. Microbiol. Biotechnol. 1: 211-219.

Hihara, Y., Kamei, A., Kanehisa, M., Kaplan, A. and Ikeuchi, M. (2001) DNA microarray analysis of cyanobacterial gene expression during acclimation to high light. Plant Cell 13: 793–806.

Hihara, Y., Sonoike, K. and Ikeuchi, M. (1998) A novel gene, pmgA, specifically regulates photosystem stoichiometry in the cyanobacterium *Synechocystis* species PCC 6803 in response to high light. Plant Physiol. 117: 1205-1216.

Hippler, M., Redding, K. and Rochaix, J.D. (1998) *Chlamydomonas* genetics, a tool for the study of bioenergetic pathways. Biochim.Biophys. Acta 1367: 1–62.

Horton ,P., Ruban, A.V. and Walters, R.G. (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol 47**:** 655-684.

Houmard, J., Capuano, V., Colombano, M.V., Coursin, T. and Tandeau de Marsac, N. (1990) Molecular characterization of the terminal energy acceptor of cyanobacterial phycobilisomes. Proc. Natl. Acad. Sci. USA 87: 2152-2156.

Howitt, C.A., Cooley, J.W., Wiskich, J.T. and Vermaas, W.F. (2001) A strain of *Synechocystis* sp. PCC 6803 without photosynthetic oxygen evolution and respiratory oxygen consumption: implications for the study of cyclic photosynthetic electron transport. Planta 214: 46-56.

Huang, L., McCluskey, M.P., Ni, H. and La Rossa, R.A. (2002) Global gene expression profiles of the cyanobacterium *Synechocystis* sp. strain PCC 6803 in response to irradiation with UV-B and white light. J. Bacteriol. 184: 6845–6858.

Hunger, K., Beckering, C.L., Wiegeshoff. F., Graumann, P.L. and Marahiel, M.A. (2006) Cold-induced putative DEAD box RNA helicases CshA and CshB are essential for cold adaptation and interact with cold shock protein B in *Bacillus subtilis*. J. Bacteriol. 188: 240–248.

Inaba, M., Suzuki, I., Szalontai, B., Kanesaki, Y., Los, D.A., Hayashi, H. and Murata, N. (2003) Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. J Biol Chem 278: 12191–12198.

Jiang, W., Fang. L. and M. Inouye. (1996) The role of the 59-end untranslated region of the mRNA for CspA, the major cold-shock protein of *Escherichia coli*, in cold-shock adaptation. J. Bacteriol. 178: 4919–4925.

Johnson, G.N., Scholes, J.D., Grime, J.P. and Horton, P. (1990) in Proceedings of the VIIIthInternational Congress on Photosynthesis.

Jones, L.W. and Myers, J. (1963) A common link between photosynthesisand respiration in a blue-green alga. Nature 199: 670–672.

Jones, P.G, Mitta, M., Kim, Y., Jiang, W. and Inouye, M. (1996) Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 93: 76–80.

Jones, P.G. and Inouye, M. (1996) RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. Mol. Microbiol. 21: 1207-1218

Jones, P.G., Van Bogelen, R.A. and Niedhardt, F.C. (1987) Induction of proteins in response to low temperature in Escherichia coli. J.Bacteriol. 169: 2092-2095.

Joshua, S. and Mullineaux, C.W. (2004) Phycobilisome diffusion is required for light-state transitions in cyanobacteria. Plant Physiol. 135: 2112-2119.

Kaan, T., Homuth, G., Mader, U., Bandow, J. and Scheweder, T. (2002) Genome-wide transcriptional profiling of the *Bacillus subtilis* cold-shock response. Microbiol. 148: 3441-3455.

Kaim, G. and Dimroth, P. (1999) ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. EMBO J. 18: 4118-4127.

Kallas, T. (1994) The cytochrome b_6f complex. In Book The Molecular Biology of Cyanobacteria. Edited by Bryant DA.pp 259-317.

Kandror, O. and Goldberg, A.L. (1997) Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. Proc. Natl. Acad. Sci. USA 94: 4978-4981.

Kandror, O., DeLeon, A. and Goldberg, A.L. (2002) Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. Proc. Natl. Acad. Sci. USA 99: 9727-9732.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu,E., Nakamura, Y., Miyajima. N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803, II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. 3: 109–136.

Kaneko, T., Nakamura, Y., Sasamoto, S., Watanabe, A., Kohara ,M., Matsumoto, M., Shimpo, S., Yamada, M. and Tabata, S. (2003) Structural analysis of four large plasmids harboring in a unicellular cyanobacterium, *Synechocystis* sp. PCC 6803. DNA Res. 10: 221–228.

Kanervo, E., Spetea, C., Nishiyama, Y., Murata, N., Andersson, B. and Aro, E.M. (2003) A cyanobacterialproteolytic system: efficiency in inducing degradation of the D1 protein of photosystem II in cyanobacteria and plants. Biochim. Biophys. Acta 1607: 131-140.

Kanesaki ,Y., Yamamoto, H, Paithoonrangsarid K, Shoumskaya M, Suzuki I, Hayashi H and Murata N (2007) Histidine kinases play important roles in the perception andsignal transduction of H_2O_2 in the cyanobacterium, *Synechocystis*. Plant J. 49: 313–324.

Kanesaki, Y., Suzuki, I., Allakhverdiev, S. I., Mikami, K. and Murata, N. (2002) Salt stress and hyperosmotic stress regulate the expression of different sets of genes in *Synechocystis* sp. PCC 6803. Biochem. Biophys. Res. Commun. 290: 339–348.

Kargul, J., Nield, J. and Barber, J. (2003) Three-dimensional reconstruction of a light-harvesting complex I-photosystem I (LHCI-PSI) supercomplex from the green alga *Chlamydomonas reinhardtii* Insights into light harvesting for PSI. J. Biol. Chem. 278: 16135-16141.

Kashino, Y., Lauber, W.M., Carroll, J.A., Wang, Q., Whitmarsh, J., Satoh, K. and Pakrasi, H.B. (2001) Proteomic analysis of a highly active photosystem II preparation from the cyanobacterium *Synechocystis* sp. PCC 6803 reveals the presence of novel polypeptides. Biochemistry 41: 8004-8012.

Kautsky, H., Appel, W. and Amann, H. (1960) Chlorophyll fluorescenz und kohlensaureassimilation. Biochemische Zeitschrift 322: 277–292.

Kawamura, M., Mimuro, M. and Fujita, Y. (1979) Quantitative relationshipbetween two reaction centers in the photosynthetic system of bluegreenalgae. Plant Cell Physiol. 20: 697–705.

Kehoe, D.M. and Grossman, A.R. (1996) Similarity of a chromatic adaptation sensor to phytochrome and ethylene receptors. Science 273: 1409-1412.

Keren, N. and Ohad, I. (1998) State transition and photoinhibition. *In* JD Rochaix, M Goldschmidt-Clermont, S Merchant, eds, The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 569–596.

Khemici, V., Toesca, I., Poljak, L., Vanzo, N.F. and Carpousis, A.J. (2004) The RNase E of Escherichia coli has at least two binding sites for DEAD-box RNA helicases: functional replacement of RhlB by RhlE. Mol. Microbiol. 54: 1422–1430.

Khrouchtchova, A., Hansson, M., Paakkarinen, V., Vainonen, J.P., Zhang, S., Jensen, P.E., Scheller, H.V., Vener, A.V., Aro, E.M. and Haldrup, A. (2005) A previously found thylakoid membrane protein of 14kDa (TMP14) is a novel subunit of plant photosystem I and is designated PSI-P. FEBS Lett. 579: 4808-4812.

Komenda, J., Hassan, H.A., Diner, B.A., Debus, R.J, Barber, J. and Nixon, P.J. (2000) Degradation of the Photosystem II D1 and D2 proteins in different strains of the cyanobacterium *Synechocystis* sp. PCC 6803 varying with respect to the type and level of psbA transcript. Plant Mol. Biol. 42:635-645.

Kovács, L., Damkjær, J., Kereïche, S., Ilioaia, C., Ruban, A.V., Boekema, E.J., Jansson, S. and Horton, P. (2006) Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts. Plant Cell 18: 3106-3120.

Kressler. D., Linder, P. and de La Cruz, J. (1999) Protein trans-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. Mol. Cell Biol. 19: 7897–7912.

Kuhl, H., Rögner, M., Van Breemen, J.F. and Boekema, E.J. (1999) Localization of cyanobacterial photosystem II donor-side subunits by electron microscopy and the supramolecular organization of photosystem II in the thylakoid membrane. Eur. J. Biochem. 266: 453-459.

Kujat, S.L. and Owttrim, G.W. (2000) Redox-regulated RNA helicase expression. Plant Physiol. 124: 703–713.

Kurisu, G., Zhang, H., Smith, J.L. and Cramer, W.A. (2003) Structure of the cytochrome b6f complex of oxygenic photosynthesis: tuning the cavity. Science 302: 1009-1014.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature 227: 680–685.

Lelivelt, M.J. and Kawula, T.H. (1995) Hsc66, an Hsp70 homolog in Escherichia coli, is induced by cold shock but not by heat shock. J. Bacteriol. 177: 4900-4907.

Lensch, M., Herrmann, R.G. and Sokolenko, A. (2001) Identification and characterization of SppA, a novel light-inducible chloroplast peptidase complex associated with thylakoid membranes. J. Biol. Chem. 276: 33645-33651.

Lill, H. and Nelson, N. (1991) The atp1 and atp2 operons of the cyanobacterium *Synechocystis* sp. PCC 6803. Plant. Mol. Biol. 17: 641-652.

Lim, J., Thomas, T. and Cavicchioli, R. (2000) Low temperature regulated DEAD-box RNA helicase from the Antarctic archaeon, *Methanococcoidesburtonii*. J. Mol. Biol. 297: 553–567.

Lindahl, M., Spetea, C., Hundal, T., Oppenheim, A.B., Adam, Z. and Andersson, B. (2000) The thylakoid FtsH peptidase plays a role in the light-induced turnover of the photosystem II D1 protein. Plant Cell 12: 419-432.

Lipinska, B., Zylicz, M. and Georgopoulos, C. (1990) The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperatures, is an endopeptidase. J. Bacteriol. 172: 1791-1797.

Liu, H-Y., Nefsky, B.S. and Walworth, N.C. (2002) The Ded1 DEAD box helicase interacts with Chk1 and Cdc2. J Biol. Chem. 277: 2637–2643.

Lorimier, R.M., Smith, R.L. and Jr, Stevens, S.E. (1991) Regulation of phycobilisomes structure and gene expression by light intensity. Plant Physiol. 98: 1003-1010.

Los, D.A. (2004) The effect of low-temperature-induced DNA supercoiling on the expression of thedesaturase genes in *Synechocystis*. Cell Mol. Biol. 50: 605-612.

Los, D.A. and Murata, N. (2004) Membrane fluidity and its roles in the perception of environmental signals. Biochim. Biophys. Acta 1666: 142–157.

Los, D.A., and Murata, N. (1999) Responses to cold shock in cyanobacteria. J. Mol. Microbiol. Biotechnol. 1: 221-230.

Los, D.A., Ray, M.K. and Murata, N. (1997) Differences in the control of the temperature-dependent expression of four genes for desaturases in *Synechocystis* sp. PCC 6803. Mol. Microbiol. 25: 1167-1175.

Los, D.A., Suzuki, I., Zinchenko, V.V. and Murata, N (2008) Stress responses in *Synechocystis*: regulated genes and regulatory systems. In *The Cyanobacteria: Molecular Biology, Genomics and Evolution*, Herrero, A., Flores, E., Eds.; Caister Academic Press: Norfolk, UK; pp. 117-157.

Lund, M.K. and Guthrie, C. (2005) The DEAD-box protein Dbp5p is required to dissociate Mex67p from exported mRNPs at the nuclear rim. Mol. Cell 20: 645–651.

MacColl, R. (1998) Cyanobacterial phycobilisomes. J. Struct. Biol. 124: 311–334.

Mann, N.H., Novac, N., Mullineaux, C.W., Newman, J., Bailey, S. and Robinson, C. (2000)Involvement of an FtsH homologue in the assembly of functional photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803. FEBS Lett. 479: 72–77

Mano, J., Miyake, C., Schreiber, U. and Asada, K. (1995) Photoactivation of the electron flow from NADPH to plastoquinone in spinach chloroplasts. Plant Cell Physiol 36: 1589–1598.

Manodori, A. and Melis, A. (1986) Cyanobacterial acclimation to photosystem Ior photosystem II light. Plant Physiol. 82: 185–189.

Mansilla, M.C., Cybulski, L.E., Albanesi, D. and De Mendoza, D. (2004) Control of membrane lipid fluidity by molecular thermosensors. J. Bacteriol. 186: 6681-6688.

McConnell, M.D., Koop, R., Vasil'ev, S. and Bruce, D. (2002) Regulation of the distribution of chlorophyll and phycobilin-absorbed excitation energy in cyanobacteria. A structure-based model for the light state transition. Plant Physiol. 130: 1201-1212.

Melis, A. (1991) Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage? Trends. Plant Sci. 4: 130-135.

Melis, A. and Brown, J.S. (1980) Stoichiometry of system I and system II reaction centers and of plastoquinone in different photosynthetic membranes. Proc. Natl. Acad. Sci. USA 77: 4712–4716.

Melis, A., Murakami, A., Nemson, J.A., Aizawa, K., Ohki, K. and Fujita, Y. (1996) Chromatic regulation in Chlamydomonas reinhardtii alters photosystem stoichiometry and improves the quantum efficiency of photosynthesis. Photosynth. Res. 47: 253–265.

Mi, H., Endo, T., Schreiber, U. and Asada, K. (1992a) Donation of electrons to the intersystem chain in the cyanobacterium *Synechococcus* sp. PCC 7002. Plant Cell Physiol. 33: 1099–1105.

Mi, H., Endo, T., Schreiber, U., Ogawa, T. and Asada, K. (1992b) Electron donation from cyclic and respiratory flows to the photosynthetic intersystem chain is mediated by pyridine nucleotide dehydrogenase in the cyanobacterium *Synechocystis* PCC 6803. Plant Cell Physiol. 33: 1233–1237.

Miyagishima, S.Y., Wolk, P. and Osteryoung K.W. (2005) Identification of cyanobacterial cell division genes by comparative and mutational analyses. Mol. Microbiol. 56:126-143.

Mohamed, A. and Jansson, C. (1989) Influence of light on accumulation of photosynthesis-specific transcripts in the cyanobacterium *Synechocystis* 6803. Plant Mol Biol. 13: 693-700.

Mohanty, P., Nellaepalli, S., Mishra, S. and Subramanyam, S. (2011) State shifts in photosynthetic organisms: Tracking traits and techniques. In: S. Itoh, P.Mohanty, K.N. Guruprasad (Eds.), Photosynthesis – Overviews on Recent Progress and Future Perspective, I.K. (Information & Knowledge) International Publishers, New Delhi, India. pp 38–59.

Müller, P., Li, X.P. and Niyogi K.K. (2001) Non-Photochemical Quenching. A response to excess light energy. Plant Physiol. 4: 1558-1566.

Mullineaux C.W. (2008) Phycobilisome-reaction centre interaction in cyanobacteria. Photosynth. Res. 95, 175-182.

Mullineaux CW, Allen JF (1990) State 1-state 2 transitions in th cyanobacterium *Synechococcus* 6301 are controlled by the redox state of the electron carriers between photosystem I and II. Photosynth Res 23: 297–311

Mullineaux, C.W. (1994) Excitation energy transfer from phycobilisomes to photosystem I in a cyanobacterial mutant lacking photosystem II. Biochim. Biophys. Acta 1184: 71-77.

Mullineaux, C.W. (1999) The thylakoid membranes of cyanobacteria: structure, dynamics and function. Aust. J. Plant Physiol. 26: 671–677.

Mullineaux, C.W. and Allen, J.F. (1986) The state 2 transition in the cyanobacterium *Synechococcus* 6301 can be driven by respiratory electron flow into the plastiquinone pool. FEBS Lett. 205: 155–160.

Mullineaux, C.W. and Emlyn-Jones, D. (2005) State transitions: an example of acclimation to low-light stress. J. Exp. Bot. 56: 389-393

Mullineaux, C.W., Tobin, M.J. and Jones, G.R. (1997) Mobility of photosynthetic complexes in thylakoid membranes. Nature 390: 421-424..

Murakami, A. and Fujita, Y. (1991) Regulation of photosystem stoichiometry in the photosynthetic system of the cyanophyte *Synechocystis* PCC 6714 in response to light intensity. Plant Cell Physiol. 32: 223-230.

Murakami, A., Fujita, Y., Nemson, J.A. and Melis, A. (1997a) Chromatic regulation in *Chlamydomonas reinhardtii*: time course of photosystem stoichiometry adjustment following a shift in growth light quality. Plant Cell Physiol. 38: 188–193

Murakami, A., Kim, S.J. and Fujita, Y. (1997b) Changes in photosystem stoichiometry in response to environmental conditions for cell growth observed with the cyanophyte *Synechocystis* PCC 6714. Plant Cell Physiol. 38: 392-397.

Muramatsu, M. and Hihara, Y. (2003) Transcriptional regulation of genes encoding subunits of photosystem I during acclimation to high-light conditions in *Synechocystis* sp. PCC 6803. Planta 216: 446–453.

Murata, N. (1969) Control of excitation transfer in photosynthesis. I. Light-induced change of chlorophyll *a* fluorescence in *Porphyridiumcruentum*. Biochim. Biophys. Acta 172: 242-251.

Murata, N. and Wada, H. (1995) Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. Biochem. J. 308: 1–8.

Murata, N. (1969) Control of excitation transfer in photosynthesis. Light-induced change of chlorophyll *a* fluorescence in *Porphyridium cruentum*. Biochim. Biophys. Acta 172: 242-251.

Nash, D., Miyao, M. and Murata, N. (1985) Heat inactivation of oxygen evolution in photosystem I1 particles and its acceleration by chloride depletion and exogenous manganese. Biochem Biophys Acta 807: 127-133.

Neisser, A., Fromwald, S., Schmatzberger, A. and Peschek, G.A. (1994) Immunological and functional localization of both F-type and P-type ATPases in cyanobacterial plasma membranes. Biochem. Biophys. Res. Commun. 200: 884-92.

Nishiyama, Y., Kovacs, E., Lee, C.B., Hayashi, H., Watanabe, T. and Murata, N. (1993) Photosynthetic adaptation to high temperature associated with thylakoid membranes of *Synechococcus* PCC7002. Plant Cell Physiol. 34: 337-343.

Ort, D.R. and Yocum, C.F. (eds) (1996) Oxygenic Photosynthesis: The Light Reactions. Dordrecht: Kluwer Academic Publishers.

Ostersetzer, O. and Adam, Z. (1997) Light-stimulated degradation of an unassembled RieskeFeS protein by a thylakoid-bound protease: the possible role of the FtsH protease. Plant Cell 9: 957-965.

Owttrim, G.W. (2006) RNA helicases and abiotic stress. Nucleic acids res. 34: 3220-3230.

Owttrim, G.W., Mandel, T., Trachsel, H., Thomas, A.A. and Kuhlemeier, C. (1994) Characterization of the tobacco eIF-4A gene family. Plant Mol. Biol. 26: 1747–1757.

Papageorgiou, G. C. (1996) The photosynthesis of cyanobacteria (blue bacteria)from the perspective of signal analysis of chlorophyll *a* fluorescence. J. Sci. Ind. Res. 55: 596–617. Photosynth. Res. 10: 7–35.

Papageorgiou, G.C. and Govindjee (2011) Photosystem II fluorescence: Slow changes - Scaling from the past. J. Photochem. Photobiol. B 104: 258-270.

Papageorgiou, G.C., Tsimilli-Michael, M. and Stamatakis, K. (2007) The fast and slow kinetics of chlorophyll *a* fluorescence induction in plants, algae and cyanobacteria: a viewpoint. Photosynth. Res. 94: 275–290.

Pedersen, P.L. and Amzel, L.M. (1993) ATP synthases.Structure, reaction center, mechanism, and regulation of one of nature's most unique machines. J. Biol. Chem. 268: 9937-9940.

Pfannschmidt, T. (2003) Chloroplast redox signals: how photosynthesis controlsits own genes. Trends Plant Sci. 8:33–41.

Pfannschmidt, T., Schutze, K., Brost, M. and Oelmuller, R. (2001)A novel mechanism of nuclear photosynthesis gene regulation by redox signals from the chloroplast during photosystem stoichiometry adjustment. J. Biol. Chem. 276: 36125–36133.

Pfannschmidt, T., Nilsson, A. and Allen, J.F. (1999) Photosynthetic control of chloroplast gene expression. Nature 397: 625–628.

Phadtare, S. (2004) Recent developments in bacterial cold-shock response. Curr Issues Mol. Biol. 6: 125–136.

Phadtare, S., and Inouye, M. (2004) Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-csp-deletion strains of Escherichia coli. J. Bacteriol. 186: 7007-7014.

Pham, X.H, Reddy, M.K., Ehtesham, N.Z., Matta, B. and Tuteja, N. (2000) A DNA helicase from *Pisumsativum* is homologous to translation initiation factor and stimulates topoisomerase I activity. Plant J. 24: 219–229.

Porankiewiz, J., Schelin, J., and Clarke, A.K. (1998) The ATP-dependent Clp protease is essential for acclimation to UV-B and low temperature in the cyanobacterium *Synechococcus*. Mol Microbiol. 29: 275.

Prakash, J.S.S., Krishna, P,S., Sirisha, K., Kanesaki, Y., Suzuki, I., Shivaji, S.and Murata, N.(2010). An RNA helicase, CrhR, regulates the low-temperature-inducible expression of heat-shock genes groES, groEL1 and groEL2 in *Synechocystis* sp. PCC 6803. Microbiol. 156: 442-451.

Prakash, J.S., Sinetova, M., Zorina, A., Kupriyanova, E., Suzuki, I., Murata, N. and Los, D.A. (2009) DNA supercoiling regulates the stress-inducible expression of genes in the cyanobacterium *Synechocystis*. Mol. Biosyst. 5: 1904-1912.

Prud'homme-Genereux, A., Beran, R.K., Iost, I., Ramey, C.S., Mackie, G.A. and Simons, R.W. (2004) Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. Mol. Microbiol. 54: 1409-14021.

Pursiheimo, S., Rintamäki, E., Baena-Gonzalez, E. and Aro, E.M. (1998) Thylakoid protein phosphorylation in evolutionally divergent species with oxygenic photosynthesis. FEBS Lett. 423: 178-182.

Purusharth, R.I., Klein, F., Sulthana, S., Jager, S., Jagannadham, M.V., Evguenieva-Hackenberg, E., Ray, M.K. and Klug, G. (2005) Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium *Pseudomonas syringae* Lz4W. J. Biol.Chem. 280: 14572–14578.

Puthiyaveetil, S. and Allen, J. F. (2008) Transients in chloroplast genetranscription. Biochem. Biophys. Res. Commun. 368: 871–874.

Py, B., Higgins, C.F., Krisch, H.M. and Carpousis, A.J. (1996).A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. Nature 3: 169-172.

Rakhimberdieva, M.G., Boichenko, V.A., Karapetyan, N.V. and Stadnichuk, I.N. (2001) Interaction of phycobilisomes with photosystem II dimers and photosystem I monomers and trimers in the cyanobacterium*Spirulinaplatensis*. Biochemistry 40: 15780-15788.

Rakhimberdieva, M.G., Elanskaya, I.V., Vermaas, W.F.J. and Karapetyan, N.V. (2010) Carotenoid-triggered energy dissipation in phycobilisomes of *Synechocystis* sp. PCC 6803 diverts excitation away from reaction centers of both photosystems Biochim. Biophys. Acta 1797: 241-249.

Rauhut, R., and Klug, G. (1999) mRNA degradation in bacteria. FEMS Microbiol. Rev. 23: 353-370.

Ray, M. K. (2006). Cold-stress response of low temperature adapted bacteria. In *Stress response: A Molecular Biology Approach, 2006,* pp. 1–23. Edited by A. S. Sreedhar and U. K. Srinivas, Research Signpost, Fort P.O., Trivandrum-695 023, Kerala, India.

Rich, P.R. and Bendall, D.S. (1980) The redox potentials of the b-type cytochromes of higher plant chloroplasts. Biochim.Biophys. Acta 591:153-161.

Richaud, C., Zabulon, G., Joder, A. and Thomas, J.C. (2001) Nitrogen or sulphur starvation differentially affects phycobilisome degradation and expression of the nblA gene in *Synechocystis* strain PCC 6803. J. Bacteriol. 183: 2989-2994.

Ro¨gner, M., Nixon, P.J. and Diner, B.A. (1990) Purification and characterization of photosystem I and photosystem II core complexes from wild-type and phycocyanin-deficient strains of the cyanobacterium *Synechocystis* PCC 6803. J. Biol. Chem. 265: 6189–6196.

Robertson, D., Woessner, J.P., Gillham, N.W. and Boynton, J.E. (1989) Molecular characterization of two point mutants in the chloroplast atpB gene of the green alga *Chlamydomonas reinhardtii* defective in assembly of the ATP synthase complex. J. Biol. Chem. 264: 2331-233.

Rocak, S. and Linder, P. (2004) DEAD-box proteins: the driving forces behind RNA metabolism. Nature Rev. Mol. Cell Biol. 5: 232–241.

Rochaix, J.D. (2011) Reprint of: Regulation of photosynthetic electron transport. Biochim. Biophys. Acta 1807: 878-886.

Rossler, O.G., Straka, A. and Stahl, H. (2001) Rearrangement of structured RNA via branch migration structures catalysed by the highly related DEAD-box proteins p68 and p72. Nucleic Acids Res. 29: 2088–2096.

Russell, N.J. and Nichols, D.S. (1999) Polyunsaturated fatty acids in marine bacteria—a dogma rewritten. Microbiology 145: 767–779.

Russell, N.J. (1990) Cold adaptation of microorganisms. Philos. Trans. R. Soc. Lond. B. Bio. Sci. 329: 595-608.

Sakamoto, T.and Bryant, D.A. (1998) Growth at low temperature causes nitrogen limitation in the cyanobacterium*Synechococcus* sp. PCC 7002. Arch. Microbiol. 169:10-9.

Sakamoto, T. and Bryant, D.A. (1997) Temperature-regulated mRNA accumulation and stabilization for fatty acid desaturase genes in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Mol. Microbiol. 23: 1281–1292.

Sanan-Mishra, N., Pham, X.H., Sopory, S.K. and Tuteja, N. (2005) Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. Proc. Natl. Acad. Sci. USA 102: 509–514.

Sandstrom, S., Ivanov, A.G., Park, Y.I., Oquist, G. and Gustafsson, P. (2002) Iron stress responses in the cyanobacterium *Synechococcus* sp. PCC7942. Physiol. Plant 16: 255-263.

Sarcina, M., Tobin, M.J. and Mullineaux, C.W. (2001) Diffusion of phycobilisomes on the thylakoid membranes of the cyanobacterium *Synechococcus* 7942. Effects of phycobilisome size, temperature, and membrane lipid composition. J. Biol. Chem. 276: 46830-46834.

Savitch, L.V., Barker-Astrom, J., Ivanov, A.G., Hurry, V., Oquist, G., Huner, N.P. and Gardestrom, P. (2001) Cold acclimation of *Arabidopsis thaliana* results in incomplete recovery of photosynthetic capacity, associated with an increased reduction of the chloroplast stroma. Planta 214: 295–303.

Schade, B., Jansen, G., Whiteway, M., Entian, K.D. and Thomas, D.Y. (2004) Cold adaptation in budding yeast. Mol. Biol. Cell 15: 5492–5502.

Schägger, H. and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. 199: 223-231.

Scheller, H.V., Jensen, P.E., Haldrup, A., Lunde, C. and Knoetzel, J. (2001) Role of subunits in eukaryotic photosystem I. Biochim.Biophys. Acta 1507: 41-60.

Schmetterer, G., Alge, D. and Gregor, W. (1994) Deletion of cytochrome c oxydase genes from the cyanobacterium *Synechocystis* sp. PCC 6803: evidence for alternative respiratory pathways. Photosynth. Res. 42: 43-50.

Schreiber, U. (2004) Pulse-Amplitude-Modulation (PAM) fluorometry and saturationpulse method: an overview. Chlorophyll a Fluorescence: A Signature of Photosynthesis, ed. Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Springer, Dordrecht. Pp. 279-319.

Schreiber, U., Schliwa, U. and Bilger, W. (1986) Continuous recording of photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth. Res.10: 51-62.

Sener, M K., Jolley, C., Ben-Shem, A., Fromme, P., Nelson, N., Croce, R. and Schulten, K (2005) Comparison of the light harvesting networks of plant and cyanobacterial photosystem I. *Biophys. J.* 89:1630-1642.

Shen, G., Boussiba, S. and Vermaas, W.F.J. (1993) *Synechocystis*sp. PCC 6803 strains lacking photosystem I and phycobilisome function. Plant Cell 5: 1853–1863

Shen, G., Zhao, J., Reimer, S.K., Antonkine, M.L., Cai, Q., Weiland, S.M., Golbeck, J.H. and Bryant, D.A. (2002)Assembly of photosystem I. Inactivation of the rubA gene encoding a membrane-associated rubredoxin in the cyanobacterium *Synechococcus* sp. PCC 7002 causes a loss of photosystem I activity. J. Biol. Chem. 277: 20343–20354.

Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K. and Yokota, A. (1998) Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. Proc. Natl. Acad. Sci. USA 95: 9705-9709.

Shneyour, A., Raison, J.K. and Smillie, R.M. (1973) The effect of temperature of the rate of photosynthetic electron transfer in chloroplasts of chilling-sensitive and chilling-resistant plants. Biochim. Biophys. Acta 292: 152-161.

Silva, P., Thompson, E., Bailey, S., Kruse, O., Mullineaux, C.W., Robinson, C., Mann, N.H. and Nixon, P.J. (2003) FtsH is involved in the early stages of repair of photosystem II in *Synechocystis* sp PCC 6803 Plant Cell 15: 2152-2164.

Silverstein, T., Cheng, L. and Allen, J.F. (1993) Chloroplast thylakoid protein phosphatase reactions are redox-independent and kinetically heterogeneous. FEBS Lett. 334: 101-105.

Sinensky, M. (1974) Homeoviscous adaptation--a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 71: 522-525.

Singleton, M. R. and Wigley, D. B. (2002) Modularity and specialization in superfamily 1 and 2 helicases. J. Bacteriol. 184: 1819–1826.

Snay-Hodge, C.A., Colot, H.V., Goldstein, A.L. and Cole, C.N. (1998) Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export. EMBO. J 17: 2663–2676.

Sonoike, K., Hihara, Y. and Ikeuchi, M. (2001) Physiological significance of the regulation of photosystem stoichiometry upon high light acclimation of *Synechocystis* sp. PCC 6803. Plant Cell Physiol. 42: 379–384.

Spiess, C., Beil, A. and Ehrmann, M. (1999) A temperature-dependent switch from chaperone to peptidase in a widely conserved heat stress protein. Cell 97: 339-347.

Spurr, A.R. (1969) A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructure Res. 26: 31-57.

Stamatakis, K., Tsimilli-Michael, M. and Papageorgiou, G.C. (2007) Fluorescence induction inthe phycobilisome-containing cyanobacterium *Synechococcus*sp PCC 7942: analysis of the slow fluorescence transient. Biochim. Biophys. Acta 1767: 766–772.

Stanier, R. Y., Kunisawa, R., Mandel, R. and Cohen-Bazire, G. (1971) Purification and properties of unicellular blue-green algae (order *chrococcales).* Bacterial Rev. 35: 171–205.

Strasser, R.J., Tsimilli-Michael, M. and Srivastava, A (2004)Analysis of the chlorophyll a fluorescence transient, in: G.C. Papageorgiou, Govindjee (Eds.), Advances in Photosynthesis and Respiration, Vol. 19, Chlorophyll Fluorescence: a Signature of Photosynthesis, Springer, Dordrecht, pp. 321–362.

Strauch, K.L., Johnson, K. and Beckwith, J. (1989) Characterization of degP, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. J. Bacteriol. 171: 2689-2696.

Strocchi, M., Ferrer, M., Timmis, K. N. and Golyshin, P. N. (2006) Low temperature-induced systems failure in *Escherichia coli*: insights from rescue by cold-adapted chaperones. Proteomics 6: 193–206.

Suzuki, I., Kanesaki, Y., Mikami, K., Kanehisa, M. and Murata, N. (2001) Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. Mol. Microbiol. 40: 235–244.

Suzuki, I., Los, D.A., Kanesaki, Y., Mikami, K. and Murata, N. (2000) The pathway for perception and transduction of low-temperature signals in *Synechocystis*. EMBO J 19: 1327–1334.

Suzuki, S., Ferjani, A., Suzuki, I. and Murata, N. (2004) The SphS-SphR two component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in *Synechocystis*. J Biol. Chem. 279: 13234–13240.

Szabo, I., Rigoni, F., Bianchetti, M., Carbonera, D., Pierantoni, F., Seraglia, R., Segalla, A. and Giacometti, G.M. (2001) Isolation and characterization of photosystem II subcomplexes from cyanobacteria lacking photosystem I. Eur. J. Biochem. 268: 5129-5134.

Tandeau de Marsac, N. and Houmard, J. (1993) Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanisms. FEMS Microbiol. Lett. 104: 119–190.

Tanner, N.K., and Linder, P. (2001) DExD/H box RNA helicases: from generic motors to specific dissociation functions. Mol. Cell: 8251–262.

Tanner, N. K.(2003) The newly identified Q motif of DEAD box helicases is involved in adenine recognition. *Cell Cycle* 2, 18–19.

Tanner, N. K., Cordin, O., Banroques, J., Doère, M. and Linder, P. (2003)The Q Motif. A newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. *Mol. Cell* 11, 127–138.

Thieringer, H.A., Jones, P.G. and Inouye, M. (1998) Cold shock and adaptation. BioEssays 20: 49-57.

Tikkanen, M., Grieco, M., Kangasjarvi, S. and Aro, E.A. (2010) Thylakoid Protein Phosphorylation in Higher Plant Chloroplasts Optimizes Electron Transfer under Fluctuating Light. Plant Physiol. 152: 723–735.

Tsimilli-Michael, M., Stamatakis, K. and Papageorgiou, G.C. (2009) Dark-to-light transition in *Synechococcus* sp PCC7942 cells studied by fluorescence kinetics assesses plastoquinone redox poise in the dark and photosystem II fluorescence component and dynamics during state 2 to state 1 transition. Photosynth. Res. 99: 243-255.

Tsunoyama, Y., Bernát, G., Dyczmons, N.G., Schneider, D. and Rögner, M. (2009) Multiple Rieske proteins enable short- and long-term light adaptation of *Synechocystis* sp. PCC 6803. J. Biol. Chem. 284: 27875-27883.

Tu, C.J., Shrager, J., Burnap, R.L., Postier, B.L. and Grossman, A.R. (2004) Consequences of a deletion in *dspA* on transcript accumulation in *Synechocystis* sp. strain PCC 6803. J. Bacteriol. 186: 3889-3902.

Tullberg, A., Alexciev, A., Pfannschmidt, T. and Allen, J.F. (2000) Photosynthetic electron flow regulates transcription of the psaB gene in pea (*Pisum sativum* L.) chloroplasts through the redox state of the plastoquinone pool. Plant Cell Physiol. 41: 1045-1054.

van Thor, J.J., Mullineaux, C.W., Matthijs, H.C.P. and Hellingwerf, K.J. (1998) Light harvesting and state transitions in cyanobacteria. Bot. Acta: 430–443.

van Waasbergen, L.G., Dolganov, N. and Grossman, A.R. (2002) *nblS*, a gene involved in controlling photosynthesis-related gene expression during high light and nutrient stress in *Synechococcus elongatus* PCC 7942. J. Bacteriol. 184: 2481-2490.

Vashisht, A.A., Pradhan, A., Tuteja, R. and Tuteja, N. (2005) Cold- and salinity stress-induced bipolar pea DNA helicase 47 is involved in protein synthesis and stimulated by phosphorylation with protein kinase C. Plant J. 44: 76–87.

Vener, A.V., van Kan, P.J., Rich, P.R., Ohad, I. and Andersson, B. (1997) Plastoquinol at the quinol oxidation site of reduced cytochrome b_6f mediates signal transduction between light and protein phosphorylation: Thylakoid protein kinase deactivation by a single-turnover flash. Proc. Natl. Acad. Sci. USA 94: 1585-1590.

Vermaas, W.F. (1998) Gene modifications and mutation mapping to study the function of photosystem II. Methods Enzymol. 297: 293–310.

Vermaas, W.F.J. (2001) Photosynthesis and respiration in cyanobacteria. In: Encyclopedia of Life Sciences, Nature Publishing Group, London, pp. 245-251.

Vinnemeier, J. and Hagemann, M. (1999) Identification of salt-regulated genes in the genome of the cyanobacterium *Synechocystis* sp. Strain PCC 6803 by subtractive RNA hybridization. Arch. Microbiol. 172: 377–386.

Wada, H. and Murata, N. (1989). *Synechocystis* PCC 6803 mutants defective in desaturation of fatty acids. Plant Cell Physiol. 30: 971–978.

Wada, H., Avelange-Macherel, M. and Murata, N. (1993) The desA gene of the cyanobacterium *Synechocystis* sp. strain PCC 6803 is the structural gene for Δ12 desaturase. J. Bacteriol.175: 6056-6058.

Wang, H.L., Postier, B.L. and Burnap, R.L. (2004) Alterations in global patterns of gene expression in *Synechocystis* sp. PCC 6803 in response to inorganic carbon limitation and the inactivation of ndhR, a LysR family regulator. J. Biol. Chem. 279: 5739–5751.

Wang, J.C. and Lynch, A.S. (1993) Transcription and DNA supercoiling. Curr. Opin. Genet. Dev. 3: 764-768.

Weis, E. (1985) Light and temperature-induced changes in the distribution of excitation energy between Photosystem I and Photosystem II in spinach leaves. Biochim. Biophys. Acta 41731: 118-126.

Westermarck, J., Weiss, C., Saffrich, R., Kast, J., Musti, A.M., Wessely, M., Ansorge, W., Seraphin, B., Wilm, M. and Valdez, B.C. (2002) The DEXD/H-box RNA helicase RHII/Gu is a co-factor for c-Jun-activated transcription. EMBO J 21: 451–460.

Whitmarsh, J. (1998) Electron transport and energy transduction. In: Raghavendra AS (ed.) *Photosynthesis: A Comprehensive Treatise*, Cambridge: Cambridge University Press. pp. 87–107.

Widger, W.R., Cramer, W.A., Herrmann, R.G. and Trebst, A.(1984) Sequence homology and structural similarity between cytochrome b of mitochondrial complex III and the chloroplast b6f complex: position of the cytochrome b hemes in the membrane. Proc. Natl. Acad. Sci. USA 81: 674-678.

Wilde, A., Härtel, H., Hübschmann, T., Hoffmann, P., Shestakov, S.V. and Börner, T. (1995) Inactivation of a *Synechocystis* sp. strain PCC 6803 genewith homology to conserved chloroplast open reading frame 184 increases the photosystem II-to-photosystem I ratio. Plant Cell 7: 649–658.

Wilde, A., Lünser, K., Ossenbühl, F., Nickelsen, J. and Börner, T. (2001) Characterization of the cyanobacterial ycf37: mutation decreases the photosystem I content. Biochem. J. 357: 211–216.

Williams, J.G.K. (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* PCC 6803. Methods Enzymol. 167: 766–778.

Wollman, F.A. (2001) State transitions reveal the dynamics and flexibility of the photosynthetic apparatus. EMBO J. 20: 3623–3630.

Yamanaka, G., Glazer, A.N. and Williams, R.C. (1980) Molecular architecture of a light-harvesting antenna.Comparison of wild type and mutant *Synechococcus* 6301 phycobilisomes. J Biol. Chem. 255: 1104-1110.

Yamanaka, K., Mitta, M. and Inouye, M. (1999) Mutation analysis of the 5' untranslated region of the cold shock cspA mRNA of *Escherichia coli*. J. Bacteriol. 181: 6284-6291.

Yu, J. and Vermaas, W.F. (1991) Synthesis and turnover of photosystem II reaction center polypeptides in cyanobacterial D2 mutants. J. Biol. Chem. 268: 7407-7413.

Yu, L., Zhoa, J., Bryant, D.A. and Golbeck, J.H. (1993) PsaE is required for *in vivo*cyclic electron flow around photosystem I in the cyanobacterium *Synechococcus* sp. PCC 7002. Plant Physiol. 103: 171–180.

Zabulon, G.; Richaud, C.; Guidi-Rontani, C. and Thomas, J.C. (2007) NblA gene expression in *Synechocystis* PCC 6803 strains lacking DspA (Hik33) and a NblR-like protein. Curr. Microbiol. 54:36-41.

Zhang, H., Kurisu, G., Smith, J.L. and Cramer, W.A. (2003) A defined protein-detergent-lipid complex for crystallization of integral membrane proteins: The cytochrome b6f complex of oxygenic photosynthesis. Proc. Natl. Acad. Sci. USA 100: 5160-5163.

Zhao, J. and Brand, J.J. (1989) Specific bleaching of phycobiliproteins from cyanobacteria and red algae at high temperature *in vivo*. Arch. Microbiol. 152: 447-452.