# 学位論文

# Molecular cloning and physiological characterization of a gene involved in acclimation to high light in cyanobacteria

# シアノバクテリアの強光順化に関わる遺伝子の単離とその生理学的解析

平成9年12月 博士(理学)申請

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## Contents

## Abbreviations

| | |
|---|---|
| Asc | ascorbic acid |
| ATP | adenosine 5'-triphosphate |
| bp | base pair(s) |
| 2,6-DCBQ | 2,6-dichlorobenzoquinone |
| DAD | diaminodurene |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| EDTA | ethylenediamine tetraacetic acid |
| EMS | ethyl methane sulfonate |
| HEPES | 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid |
| kDa | kilo dalton |
| HQNO | 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide |
| LHC | light harvesting complex |
| LDS | lithium dodecyl sulfate |
| MV | methyl viologen |
| NADPH | nicotinamide adenine dinucleotide reduced form |
| OD730 | optical density at 730 nm |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| PQ | plastoquinone |
| PSI | photosystem I |
| PSII | photosystem II |
| QA | primary electron accepting plastoquinone of PSII |
| RT-PCR | reverse transcription-polymerase chain reaction |
| RuBisCO | ribulose 1,5-bis-phosphate carboxylase/oxygenase |

| | |
|---|---|
| SDS | sodium dodecyl sulfate |
| TES | *N*-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid |
| TMPD | *N*,*N*,*N*',*N*'-tetramethyl-*p*-phenylenediamine |
| Tris | tris(hydroxylmethyl)aminomethane |

## General Introduction

Growth of photosynthetic organisms is directly and dramatically influenced by light environment since these organisms obtain nearly all of the carbon and chemical energy needed for the growth through photosynthesis. However, in natural environment, both light quantity and quality are not constant: they vary seasonally, diurnally, and spatially. Typical examples of a biased light environment can be seen in the ocean. The underwater light climate ranges from near sun light at the sea surface to dim light in the deep sea. In an extreme case, algae or cyanobacteria are found more than 100 m below the sea surface, where the light intensity is less than 1% of sun light (Doty et al., 1974). Moreover, spectral properties of the light are greatly modified by passing through the sea water. Blue and green light are lost due to scattering by microorganisms and/or floating particles. Red light is absorbed by water itself. Thus, in the open ocean light in the blue-green wavelength region (maximal wavelength around 480 nm ) is most penetrating and utilized for photosynthesis, while in the coastal seas only light in the orange region (around 560 nm) remains available depending on the extent of scattering (Jerlov, 1976). Another example is a variation of the light environment within a deep forest. In a tropical rainforest in northern Australia, plants on the forest floor receive less than 0.5% of the irradiance experienced above the plant canopy. Their light environment comprises very low intensity diffuse light (~ 60%) which is greatly enriched in far-red light, and direct intense sunflecks (~40%) of duration from 15 s to 5 min (Björkman and Ludlow, 1972).

*Short-term adaptation and long-term adaptation (acclimation)*

Due to their extremely limited abilities to choose or change their surroundings, photosynthetic organisms must cope with such a varying environment by at least two regulatory mechanisms: "short-term adaptation" and "long-term adaptation". "Short-term adaptation" is a measure to cope with sudden and brief changes of the light environment. It is represented by the state transition which is a regulatory mechanism that controls the distribution of excitation energy transfer from the light-harvesting antenna complex to the two photosystems (Fork and Satoh, 1986; Fujita et al., 1994). This regulation occurs through phosphorylation of the antenna complex without de novo synthesis of cellular components. Thus, it can occur rather rapidly and usually completes within several minutes or less. On the other hand, "long-term adaptation" occurs more slowly over a period of hours or days, since it is achieved by changing the composition, function and structure of the photosynthetic apparatus. This regulation, which is also called acclimation, seems more important than "short-term adaptation" in order to grow under various light conditions. It includes the wide range of biological responses whose mechanisms have not been well elucidated at molecular level. I focused on this phenomenon, acclimation to light environment, in my thesis.

*Acclimative responses to light environment*

The study on acclimation to light environment was started by Björkman, who compared the composition of plants growing in shaded habitats (shade plants) and of plants growing in open sunny habitats (sun plants) (Björkman et al., 1972). Then, the effects of growth light

environment on the photosynthetic apparatus and on the photosynthetic rate have been clarified (Boardman, 1977; Björkman, 1981; Anderson et al., 1988). For example, shade plants have larger chloroplasts with much greater area of thylakoid membranes than sun plants have. Further, the shade plants have more chlorophyll per chloroplast, more chlorophyll *a*/*b*-antenna proteins, less chlorophyll *a*-proteins of the core of both photosystems, lower PSII/PSI ratio and smaller photosynthetic capacities, which are saturated at lower irradiance, than sun plants (Anderson et al., 1988). These tendency can be seen not only between the sun and shade plants, but also between chloroplasts which locate in sunny and shade positions of a single plant or in sunny and shade sides of a single leaf (Terashima and Inoue, 1985). Such acclimative responses seem to play a critical role in balancing electron flow between the two photosystems or the energy supply (light-harvesting and electron transport) and consumption ($CO_2$ fixation).

*Primary targets of the acclimative responses*

There seems to be several critical regulatory steps for acclimation to light environment. Photosynthetic properties of organisms grown under conditions of different light quality or light quantity have been thoroughly investigated, and it is becoming clear that the regulation is achieved mainly by biosynthesis or degradation of the photosynthetic components. Under light-limiting conditions, antenna pigments are selectively accumulated for maximal light capture. For example, the amount of LHCII, light-harvesting chlorophyll *a*/*b* complex associated with PSII in higher plants, highly increases upon the shift to low light. The features of shade plants such as

high chlorophyll content per chloroplast and low photosystem content per chlorophyll are derived from the accumulation of LHCII at low light. The accumulation is regulated at transcriptional level (Escoubas et al., 1995) and by biosynthesis of chlorophyll *b* (Preiss and Thornber, 1995), although the light-responsive factor(s) have not been identified yet. Under light-saturating conditions, where large antenna size is no more required, a major part of LHCII proteins is degraded. It was recently shown that an unidentified protease, which is associated with the thylakoid membrane, breaks down 25 kDa subunit of LHCII which is phosphorylated and migrates from its functional site to the mobile pool of LHCII (Lindahl et al., 1995; Andersson and Aro, 1997). In the course of the acclimation which changes the antenna size of PSII drastically, the photosystem stoichiometry also needs to be regulated for the balanced excitation between two photosystems. In cyanobacteria, assembly of the PSI complex was shown to be the critical step for regulation of photosystem stoichiometry (Kawamura et al., 1979; Fujita et al., 1988), although molecular mechanism for the regulation is totally unknown. In contrast to the case of LHCII, RuBisCO, the rate-limiting enzyme for $CO_2$ fixation, is accumulated under light-saturating conditions to balance with high photochemical activities (Björkman 1981). Transcription of the *rbcS* for the small subunit of RuBisCO seems to be regulated by various DNA-binding proteins such as GT-1 or B2F, which are known to bind to the light-responsive boxes (Perisic and Lam, 1992; Gilmartin et al., 1992). Again it is not clear whether or not these factors are directly involved in the light acclimation. The scavenging system for reactive oxygen species is also important in acclimation to high light. mRNA levels and activities of enzymes such as

superoxide dismutase and ascorbate peroxidase are known to be up-regulated upon the shift to high light (Mittler and Tel-Or, 1991; Karpinski et al., 1997).

*Inter-photosystem electron carriers as a possible redox sensor for acclimative responses*

To dissect the complicated regulatory mechanisms for accumulation of photosynthetic components in response to different light conditions, it is also essential to study how the change of the light environment is recognized. Up to the present, much effort has been devoted to the problem from the physiological point of view. An increasing number of reports have proposed that acclimation responses are induced by redox change of inter-photosystem electron carriers. Huner et al. (1996) suggested that induction of acclimative change is closely related to the redox state of the acceptor side of PSII, designated "excitation pressure of PSII", which is theoretically determined by the balance between temperature-independent photochemical reaction and temperature-dependent biochemical reactions under different conditions of light and temperature. For an example, in a green algae, *Chlorella vulgaris*, various conditions to give high PSII excitation pressure induces the same set of responses: higher light-saturated rates of $O_2$ evolution, a lower epoxidation state of the xanthophyll cycle, a lower total chlorophyll per cell, a higher ratio of chlorophyll *a*/*b* and a lower abundance of LHCII polypeptides (Maxwell et al., 1995). They proposed that excitation pressure of PSII is involved in signal transduction for acclimation universally in photosynthetic organisms including cyanobacteria and higher plants (Gray et al., 1996; Huner et al., 1996).

Escoubas et al. (1995) suggested that transcription of the genes encoding LHCII was regulated by the redox state of the PQ pool in a green algae, *Dunaliella tertiolecta*. When PQ pool is reduced under high light conditions, expression of the genes is repressed leading to the decrease in antennae size of PSII. Another example of transcriptional regulation by PQ pool is proposed for cytosolic ascorbate peroxidase in *Arabidopsis* (Karpinski et al., 1997). Ascorbate peroxidase, which is the key enzyme involved in $H_2O_2$ scavenging, is known to protect the photosynthetic apparatus from oxidative damage under excess light conditions. The levels of its transcripts increase when PQ pool is reduced and vice versa. Murakami and Fujita (1993) observed that HQNO, the inhibitor of oxidation of cytochrome *b*6 in cytochrome *b*6/*f* complex, inhibited the preferential accumulation of PSI complex under light absorbed mainly by PSII, while not under light absorbed by PSI in a cyanobacterium, *Synechocystis* sp. PCC 6714. They proposed that the redox state of cytochrome *b*6, which is closely linked to the inter-photosystem electron flow, is monitored for regulation of photosystem stoichiometry. On the other hand, Vener et al. (1997) showed that the presence of plastoquinol in its reduced form at the $Q_O$ site of cytochrome *b*6/*f* complex is involved in activation of thylakoid protein kinases which are believed to play an important part in acclimation processes via phosphorylation of proteins such as LHCII and D1 (Allen and Nilsson, 1997; Gal et al., 1997). Despite a number of views about sensing sites mentioned above, there is no consensus due to lack of decisive evidence of the molecular mechanisms.

*Another candidates for sensors for acclimative responses*

There are some reports that acclimative responses are regulated by factors besides the redox state of inter-photosystem electron carriers. By the observation that chlorophyll *b*-deficient mutant of barley and phycobilisome-deficient mutant of a cyanobacterium *Synechococcus* sp. PCC 7002 could not change the photosystem stoichiometry under different light qualities, it was suggested that light-harvesting pigments of the photosystems themselves act as light quality receptors (Kim et al., 1993). Various photoreceptors such as phytochrome and blue-light receptor may be also involved in acclimating processes since it is established that they play a vital role in the regulation of gene expression at many levels during development (Kloppstech, 1997). However, observations that phytochrome-deficient mutants could alter photosystem stoichiometry in response to both light quality and intensity indicate that phytochrome cannot be directly involved in these responses (Smith et al., 1993; Walters and Horton, 1995). On the other hand, several lines of evidence suggest that the blue-light receptor (Kaufman, 1993) may be involved in the regulation of photosystem stoichiometry (Walters and Horton, 1995).

Utilization of inter-photosystem electron carriers as sensors for redox regulation of acclimative responses is now becoming consensus and it seems very reasonable since the redox state of inter-photosystem electron carriers always reflects imbalance between photosystems or between energy supply and consumption. Moreover, specific photoreceptor(s) must be involved in the acclimation to light. In fact, cyanobacteria and higher plants seem to possess many photoreceptors (Kaneko et al., 1996; Mathews and Sharrock, 1997; Cashmore, 1997). Thus, the inter-photosystem electron carriers

and/or photoreceptors may participate in several signal transduction pathways for acclimation and may crosstalk each other, although nothing is known other than physiological observations at the moment.

In order to challenge such important but complicated problems, we must employ a method of molecular biology more vigorously and make efforts to find factors which are missing links in signal transduction pathways. In this study, I discovered *pmgA*, a novel gene identified as a regulatory factor in acclimation to high light. In Chapter 1, I will tell about a novel mutant phenotype of *pmgA* and its molecular cloning. In Chapter 2, by physiological approach, I will show that *pmgA* modulates photosystem stoichiometry upon the shift to high light condition. And in Chapter 3, I will demonstrate the ecological significance of *pmgA*-mediated acclimation from the evolutionary point of view.

# Chapter I

## A novel gene, *pmgA*, is essential for photomixotrophic growth but suppresses photoautotrophic growth.

## Introduction

Acclimation of the photosynthetic apparatus to various light environments is indispensable to photosynthetic organisms. It is well established that structure and function of the photosynthetic apparatus are dynamically modulated in response to changes of light intensity and/or quality. However, few studies have been devoted to a molecular approach to identify genes involved in regulation of such acclimative responses. So far, regulatory factors of the complementary chromatic adaptation have been identified from a cyanobacterium, *Fremyella diplosiphon*, having inducible genes for phycocyanin and phycoerythrin. Several genes such as *rcaC*, *rcaD* and *rcaE*, which encode a possible photoreceptor and signal transduction components, have been identified based on the characterization of mutant phenotypes (Chiang et al., 1993; Grossman et al., 1994; Kehoe and Grossman, 1996). Practically, cyanobacteria are much more convenient organisms than higher plants for gene cloning and manipulation.

"Glucose-tolerant" strain of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Williams, 1988) has been widely used as a model organism for the study of oxygenic photosynthesis because it has several advantages for molecular biology. First, it can be transformed by exogenously added homologous DNA (Grigorieva and Shestakov, 1982), so that gene manipulation is very easy. Second, it can grow using glucose as a carbon source (Rippka, 1972). The ability to grow heterotrophically allows us to create photosynthesis-deficient mutants (Vermaas, 1993). Third, the sequence determination of the entire genome has been completed very recently (Kaneko et al., 1996), though the data had not been open yet when I

performed DNA sequence analysis in this work. I took advantage of *Synechocystis* sp. PCC 6803 to identify genetic factor(s) involved in the acclimation. In the course of screening of EMS-treated cells to obtain mutants with different light sensitivity, I found that "wild-type" cells could be separated into two distinct phenotypes which differed in growth rate especially under high light or photomixotrophic conditions.

I report here the identification of a gene responsible for these growth properties and demonstrate that mutation of the gene (*pmgA*) leads to novel stimulation of photoautotrophic growth.

## Materials and Methods

*Strains and culture conditions*

A glucose-tolerant wild type strain of *Synechocystis* sp. PCC 6803 (Williams, 1988) and mutants were grown in BG-11 medium (Stanier et al., 1971) with 5 mM TES-KOH (pH 8.2). Solid medium was supplemented with 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate. To support photomixotrophic growth, glucose was added to a concentration of 5 mM. To maintain gene-disrupted mutants, 20 μg/ml spectinomycin was added. Unless stated otherwise, cultures were grown at 32°C under continuous illumination provided by fluorescent lamps at 50 $\mu Em^{-2}s^{-1}$. Liquid cultures were bubbled with air containing 1.0% (v/v) $CO_2$. Cell density in liquid culture was determined by measuring scattering as optical density at 730 nm ($OD_{730}$) with a spectrophotometer UV-160A (Shimadzu, Kyoto, Japan). Both WS and WL cells gave practically the same $1x10^8$ cells/ml at 1.0 $OD_{730}$.

*Escherichia coli* strains DH5αF' and INVαF' (Invitrogen, San Diego, USA) were grown in Luria-Bertani liquid or agar-solidified medium with 100 μg/ml ampicilin at 37°C.

*Preparation of nucleic acids*

To isolate genomic DNA, *Synechocystis* cells were washed with 5 mM EDTA and then treated with saturated NaI at 37°C for 30 min. After dilution, cells were precipitated and resuspended in 50 mM Tris-HCl (pH 8.0) and 20 mM EDTA. Cells were first incubated with 100 μg/ml ribonuclease and 4 mg/ml lysozyme at 37°C for 45 min and then lysed with

0.5% (w/v) SDS and 200 μg/ml proteinase K at 55°C overnight. DNA was extracted several times with phenol-chloroform and precipitated with ethanol.

Genomic libraries of a glucose-tolerant wild-type constructed in pUC18 were a kind gift from Dr. J.G.K.Williams at E.I. du Pont de Nemours & Co. (Wilmington, USA). Library DNAs were fractionated by electrophoresis in a 0.8% low melting agarose gel in 89 mM Tris, 89 mM Boric acid and 2 mM EDTA (pH 8.0). The gel containing DNA was sliced into pieces and melted at 65°C. DNA was extracted with phenol-chloroform.

*Complementation of WL cells*

Complementation was performed according to Dzelzkalns and Bogorad (1988) with slight modifications. One ml culture of WL cells grown to mid-log phase was spread without a top agar on BG11 plates supplemented with glucose. Each one μl of genomic DNA or DNA libraries in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA was directly applied onto the surface of the plate. The plate was placed under dim light at 32°C for a day, then wrapped with parafilm and transferred in the light of 50 $\mu Em^{-2}s^{-1}$. Transformants capable of growing under photomixotrophic conditions were detected in 5 to 7 d.

*DNA sequence analysis*

A 3.1 kbp *Eco*RI/*Hin*dIII fragment of the complementing clone was subcloned into pBluescript II $SK^+$. Bidirectional, exonuclease III-generated deletions of the plasmid were prepared according to the

manufacturer's instructions (TAKARA, Shiga, Japan). Nucleotide sequences were determined by the dye-terminator fluorescence detection method, using a Model 373S sequencer (Applied Biosystems, San Jose, USA). GenBank and EMBL databases were searched using BLAST program (Altschul et al., 1990).

Sequence of the region harboring the mutation site was determined in various strains by PCR cloning. For PCR cloning, 913 bp fragments containing *pmgA* were amplified with Primer 1 (5'-CATATGGTAGCGGCCAGCAC-3') and Primer 2 (5'-ATGGTGATGTAGCGCCTA-3') at 93°C for 30 sec, 57°C for 2 min and 72°C for 2 min for 30 cycles and at 72°C for 10 min for a final step. The fragments were cloned into a pCRII vector (Invitrogen) according to the manufacturer's instructions. More than two clones were sequenced to eliminate a PCR error.

*Construction of gene-disrupted mutants*

Gene-disrupted mutants of *Synechocystis* were generated by transformation of pBluescript containing the 3.1 kbp *Eco*RI/*Hin*dIII fragment in which a spectinomycin-resistance cartridge was inserted at the *Nhe*I site (for disruption of ORF1), the *Nsi*I site (for *pmgA*) and the *Xcm*I site (for ORF3). Transformants were selected in the minimal BG11 medium containing 10 μg/ml spectinomycin.

## Results

*Characterization of WS and WL phenotype*

I have tried to obtain mutants with enhanced light sensitivity or tolerance by EMS mutagenesis for half a year. However, I could not get any mutants with distinct phenotype. In the course of trial and error of the mutagenesis, I noticed that the wild-type strain kept in our laboratory could be divided into two subgroups: one produced slightly larger colonies than the other on minimal plates in the light (Fig. I-1). This may reflect the difference in light sensitivity between the two types of cells. I established several cell lines from both subgroups and named ones with larger colonies and with smaller colonies WL (wild type-large) and WS (wild type-small), respectively. Figure I-2 shows their growth properties under various conditions: light intensity, temperature, glucose and/or DCMU. Since colony morphology of two subgroups was indistinguishable, we used the colony size as an index of growth rate. Under photoautotrophic conditions at low light intensity (20 $\mu Em^{-2}s^{-1}$), they showed similar growth properties (Fig. I-2A). Under high light conditions (370 $\mu Em^{-2}s^{-1}$), WL grew at much faster rate than WS (Fig. I-2B). Colonies of both types turned yellowish green from normal blue green, due to the high light stress (Narusaka et al., 1996). When grown in the light at 18°C, the lowest permissive for *Synechocystis*, WL grew faster than WS, although both produced rather heterogeneous colonies (Fig. I-2C). The heterogeneity suggests that some additional factor(s) are involved in the photoautotrophic growth at 18°C. On the contrary, growth of WL was severely suppressed under mixotrophic conditions with 5 mM glucose and light (>50 $\mu Em^{-2}s^{-1}$)

at 32°C (Fig. I-2D), while growth of WS in light was slightly enhanced by glucose. It should be noted that this suppressed growth of WL cells was observed only when plates were wrapped with parafilm. The suppression was not observed when 10 mM DCMU was included to support photoheterotrophic growth (Fig. I-2E), indicating that WL cells were not sensitive to glucose itself.

*Complementation of WL phenotype*

To identify a mutation responsible for WL and WS phenotypes, I tried complementation according to the method of Dzelzkalns and Bogorad (1988). I chose the photomixotrophic conditions for complementation of the WL cells with genomic DNA from WS cells. Not only the genomic DNA from WS but also genomic libraries constructed from the glucose-tolerant wild-type cells before 1988 successfully complemented WL cells to give green colonies in the lawn of bleached cells when glucose-containing plates wrapped with parafilm were incubated in the light (Fig. I-3). A high molecular weight fraction of the library could complement WL cells. Then, I screened 400 plasmid clones from the fraction by complementation and obtained a clear positive clone containing a 14 kbp genomic insert. Restriction digestion revealed that a 3.1 kbp *Eco*RI/*Hin*dIII fragment was enough for complementation (Fig. I-4). We also confirmed that transformants showed growth properties practically the same as WS cells under various conditions.

*Nucleotide sequence and mutation site*

Nucleotide sequence of the 3.1 kbp *Eco*RI/*Hin*dIII fragment from the glucose-tolerant strain was determined by sequencing a series of deletion clones on both strands. Considering codon usage preference in *Synechocystis* sp. PCC 6803, three ORFs with preceding in-frame termination codons were detected (Fig. I-4). A deduced product of ORF1 consisting of 463 amino acid significantly resembled exo-endonuclease of yeast (Chow et al., 1992) or methyltransferase of *Escherichia coli* (Gustafsson et al., 1991). ORF2 encoded a 23-kDa polypeptide of 204 amino acid residues, while ORF3 encoded a 19-kDa polypeptide of 176 amino acid residues. These two ORFs had no obvious homology with known genes or hypothetical genes in the databases.

To localize the mutation site, further complementation was performed with the deletion clones used for sequencing. Although complementation with very short fragments was not so efficient due to the difficulty in double recombination, the mutation site was confined to a 415 bp region, which almost corresponded to ORF2 (Fig. I-4). A 913 bp DNA including this region was amplified by PCR from the genomic DNA of WL as a template, cloned into a pCRII vector and sequenced. Only a substitution of T for C at position 193 was consistently found in all PCR clones when compared with the nucleotide sequence of the 3.1 kbp *Eco*RI/*Hin*dIII fragment . This base substitution converts Leu at position 65 of the ORF2 product to Phe (Fig. I-5). On the other hand, sequences of PCR clones from the WS genome were the same as the 3.1 kbp *Eco*RI/*Hin*dIII fragment, indicating that WL differed in the single base from WS. The original motile strain of PCC 6803 and our old stock of wild type, which was derived from the glucose-tolerant strain (stored in 1991) carried the same sequence as WS. These

results suggest that the WL-type ORF2 dominated rather recently. However, it may not necessarily mean that a C at 193 is the original and functional genotype, because all the strains including the original PCC strain had been cultured more or less under laboratory conditions.

*Construction of ORF2-disrupted mutants*

In order to determine which type of ORF2 expressed a functional product, insertional inactivation mutants with a spectinomycin-resistance cartridge were constructed (Fig. I-5). Mutants were successively cultured with 10 μg/ml spectinomycin for 2 months under photoautotrophic conditions at 20 $\mu Em^{-2}s^{-1}$ and complete segregation was verified by lack of PCR amplification of the wild-type fragment (Fig. I-6). Under photoautotrophic conditions at low light (20 $\mu Em^{-2}s^{-1}$, Fig. I-7C), They could grow normally like WS and WL (Fig. I-7A,B). At high light (370 $\mu Em^{-2}s^{-1}$) the mutants (Fig. I-7F) grew much faster than WS (Fig. I-7D). Under photomixotrophic conditions their growth was severely suppressed even without parafilm (Fig. I-7I). These results indicate that WS and the original glucose-tolerant strains carry functional ORF2 and suggest that the base substitution in the WL cells resulted in partial inactivation of the *pmgA* product. It should be noted that disruption of ORF1 or ORF3 with the same cartridge did not produce the WL-like phenotype (data not shown), ruling out their involvement in the growth.

## Discussion

I demonstrated that functional ORF2 was indispensable for the photomixotrophic growth of *Synechocystis* sp. PCC 6803. Hence, I designated ORF2 *pmgA* (photomixotrophic growth), although we do not know exact function of the gene product. The WL cells and *pmgA*-disruptant showed the unusual phenotype that they grow faster than wild type (WS type), especially under high light conditions.

Until now, several mutants with phenotypes superior to wild type under some specific conditions were reported in cyanobacteria. A directed mutant of *Synechococcus* sp. PCC 7942 possessing only form II of the D1 protein of PSII was shown to be more resistant to photoinhibition than wild type (Krupa et al., 1991). However, the mutant had a slower growth rate than wild type under normal light conditions and it was concluded that the enhanced resistance to photoinhibition was not advantageous for optimal growth. By site-specific mutagenesis of the D1 protein of *Synechocystis* sp. PCC 6803, mutants which were less photoinhibited than wild type were obtained (Mäenpää et al., 1993). Again under normal growth conditions, the growth rate of the mutants was not superior to that of wild type. Non-bleaching mutants were isolated under conditions of nutrient starvation (Collier and Grossman, 1994) and high light (Narusaka et al., 1996). Though there are few published data about their growth rates, the latter was observed to have lower photosynthetic efficiency than wild type under normal growth conditions (Y. Narusaka, personal communication). In contrast to these, our WL cells and the *pmgA*-disrupted mutants grew better than wild type even in the medium light of 50 $\mu Em^{-2}s^{-1}$ and much better at

higher light intensity (>100 $\mu Em^{-2}s^{-1}$). To our knowledge, they are the first in cyanobacteria to show improved photoautotrophic reproduction compared with wild type.

While WL cells and *pmgA* disruptant preferred to the photoautotrophic conditions, they did not tolerate the photomixotrophic conditions. Several glucose sensitive mutants of *Synechocystis* sp. PCC 6803 have been known but none of them were comparable to the *pmgA* mutants. Gene disruption of PSII-K (M.Ikeuchi, unpublished result) and ferredoxin-dependent glutamate synthase (K.Terauchi, M.Ikeuchi and M.Ohmori, unpublished result) caused sensitivity to photomixotrophic conditions. However, photoautotrophic growth of these mutants was also suppressed to some extent. A disrupted mutant of *icfG* gene was reported to be sensitive to glucose under limitation of inorganic carbon (Beuf et al., 1994). Unlike the *pmgA* mutants, the suppression by glucose was independent of light illumination. Further, the *icfG* mutant was insensitive to glucose under high $CO_2$ conditions. These differences seem to show that *icfG* and *pmgA* work independently. The sensitive site of the *pmgA* mutants under photomixotrophic conditions has not been elucidated yet. However, the fact that the mutants could grow with glucose at the initial phase implicated that accumulation of glucose itself or some glucose-mediated stresses in the cell was a prerequisite for the following suppression of growth. Combination of photosynthesis and glucose-induced respiration might lead to some imbalance in cellular metabolisms in the mutants. The specific effect of parafilm on the WL cells might be accounted for by enhancement of this imbalance due to blockage of certain gas exchange. In a liquid culture, glucose-induced suppression was not so clear as on the agar

plate. Probably this was because the mutant cells did not accumulate glucose sufficient for the suppression at an early stage of growth and self-shading of cells reduced the incident light energy at later stage in the liquid. In fact, preliminary experiments showed that photomixotrophic growth of the mutant in the liquid was completely prevented under very high light (>500 $\mu Em^{-2}s^{-1}$).

The nucleotide sequence of *pmgA* does not show significant homology with known genes even in the latest detabases. Since the deduced amino acid sequence does not have a putative transmembrane domain, it is probably a soluble protein. It seems unlikely that *pmgA* is a gene for an enzyme involved in major metabolic pathways judging from normal growth under photoautotrophic and photoheterotrophic conditions. There were some suggestive features in the amino acid sequence of *pmgA*. Segments from 115-Ile to 123-Asp and from 151-Gly to 159-Gln were significantly homologous to G1, G2 box of sensory transduction histidine kinases of two-component regulatory system, respectively (Fig. I-5). Especially, these domains resembled those of two hypothetical proteins of *Synechocystis* sp. PCC 6803 (*sll 0337, slr 0210*), which were assumed to be histidine kinases (Kaneko et al. 1996), although *pmgA* does not have a conserved His residue. It is also marked that there were three clusters of Cys-Pro with a total of 7 Cys residues. These features might be involved in the unidentified function of *pmgA*.

I showed in this chapter that *pmgA* product is essential for photomixotrophic growth, whereas it represses photoautotrophic growth in wild-type *Synechocystis* PCC 6803. The observation may indicate that *pmgA* involves in acclimation processes to different light intensities and/or

different growth conditions. To clarify the role of *pmgA* , I will characterize the phenotype of *pmgA* mutants more precisely in Chapter 2.

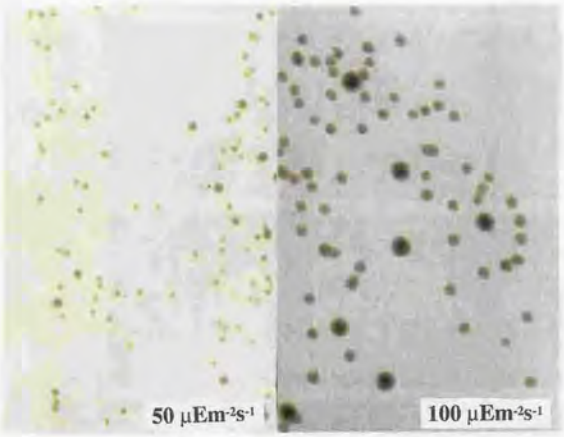



Fig. I-1 *The heterogeneity of the wild-type strain of Synechocystis sp. PCC 6803 on minimal plates in the light.*

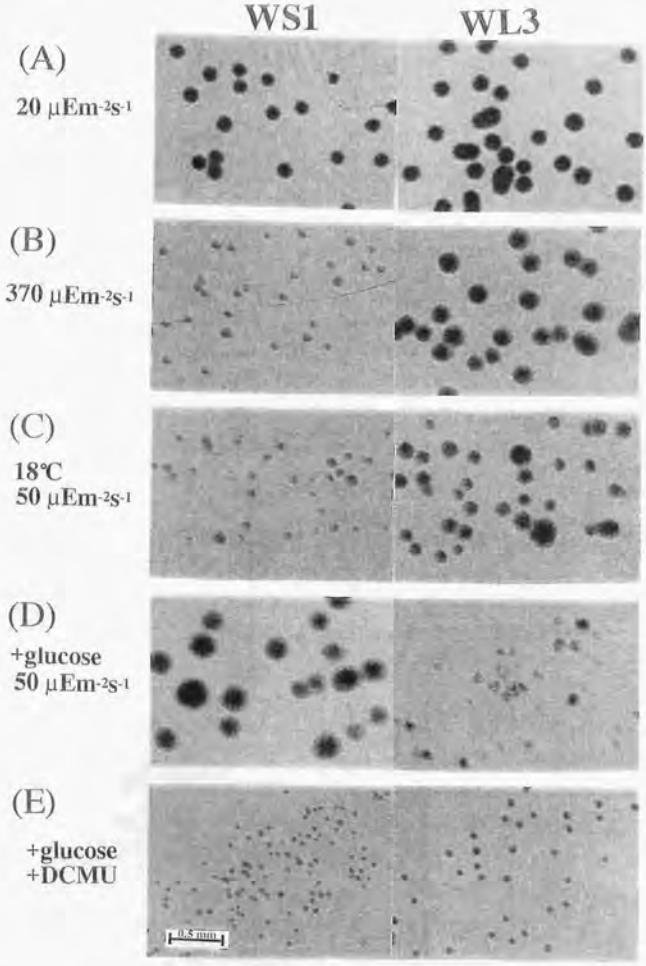



Fig. I-2 *Growth properties of WS and WL cells under various conditions as demonstrated by colony size.*

WS and WL cells were grown in the light at 20 $\mu Em^{-2}s^{-1}$ for 4 days (A), 370 $\mu Em^{-2}s^{-1}$ for 3 days (B), 50 $\mu Em^{-2}s^{-1}$ at 18℃ for 10 days (C), 50 $\mu Em^{-2}s^{-1}$ with glucose on the plate wrapped with parafilm for 5 days (D) and 50 $\mu Em^{-2}s^{-1}$ with glucose and DCMU for 5 days. Note the difference in incubation time for each condition.

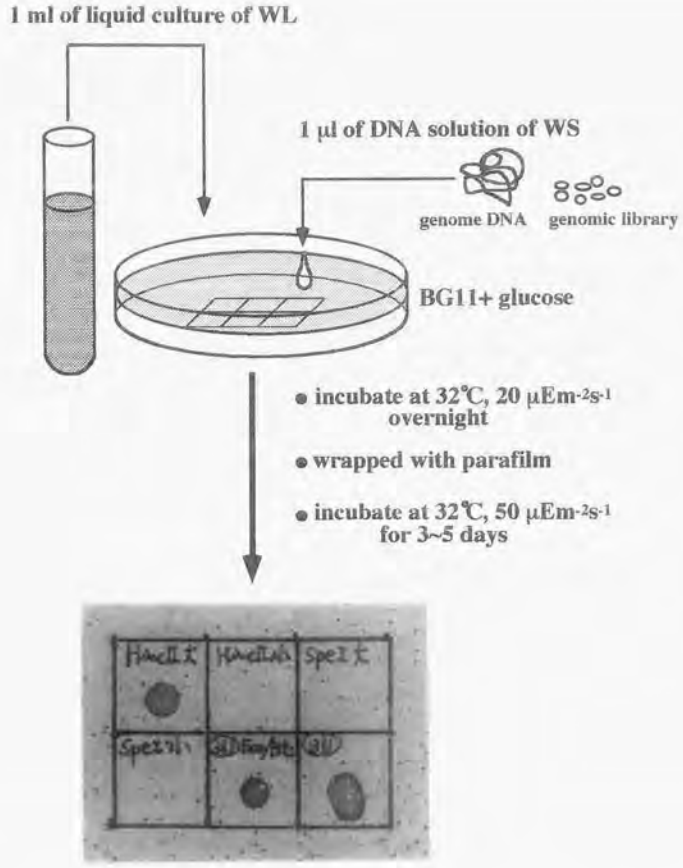



Fig. I-3 *A scheme of complementation of the WL phenotype with DNA from the WS strain under photomixotrophic conditions.*

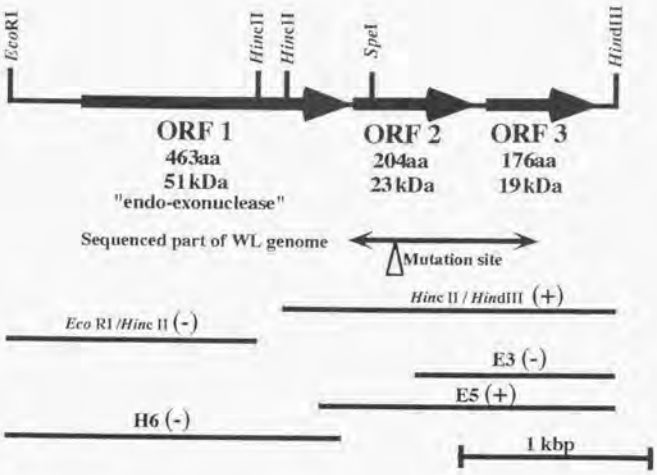
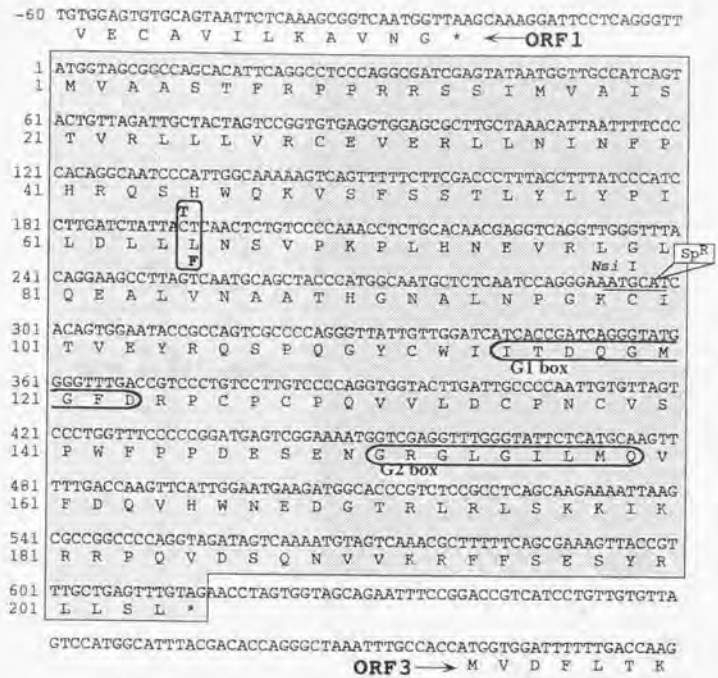



Fig. I-4 *Schematic representation of a EcoRI/HindIII fragment capable of complementing the WL phenotype.*

Recognition sites for restriction endonucleases are shown at the top. The lengths and the orientations of the three ORFs are indicated by bold arrows. The sequenced part of the WL genome and the mutation site are shown with a thin arrow and an open triangle, respectively. Restriction fragments and deletion clones used for complementation are shown in the lower part of the figure. E3 and E5 were generated by deletion from the *Eco*RI end and H6 was generated by deletion from the *Hin*dIII end of the *Eco*RI/*Hin*dIII fragment. (+) or (–) indicates the capability of complementation.




| | G1 box | G2 box |
|---|---|---|
| pmgA | ITDQGMGFD | GRGLGILMQ |
| s110337 | IRDQGLGFQ | GSGLGLAIA |
| s1r0210 | VIDTGIGID | GTGLGLALS |
| EnvZ | VEDDGPGIA | GTGLGLAIV |

Fig. I-5 *Nucleotide sequence and deduced amino acid sequence of pmgA and its flanking region.*

The full-length sequence of *pmgA* is shown in the upper panel. The deduced amino acid sequence is shown below the nucleotide sequence. The mutation site in WL is shown by bold letters in the ellipse. The *Nsi*I site at which the spectinomycin-resistance cartridge ($Sp^R$) was inserted is underlined. The 3' region of ORF1 and the 5' region of ORF3 are also indicated. Regions homologous to G1 and G2 box of histidine kinases of the two-component regulatory system are indicated in the upper panel and their sequence allignment is shown in the lower panel.

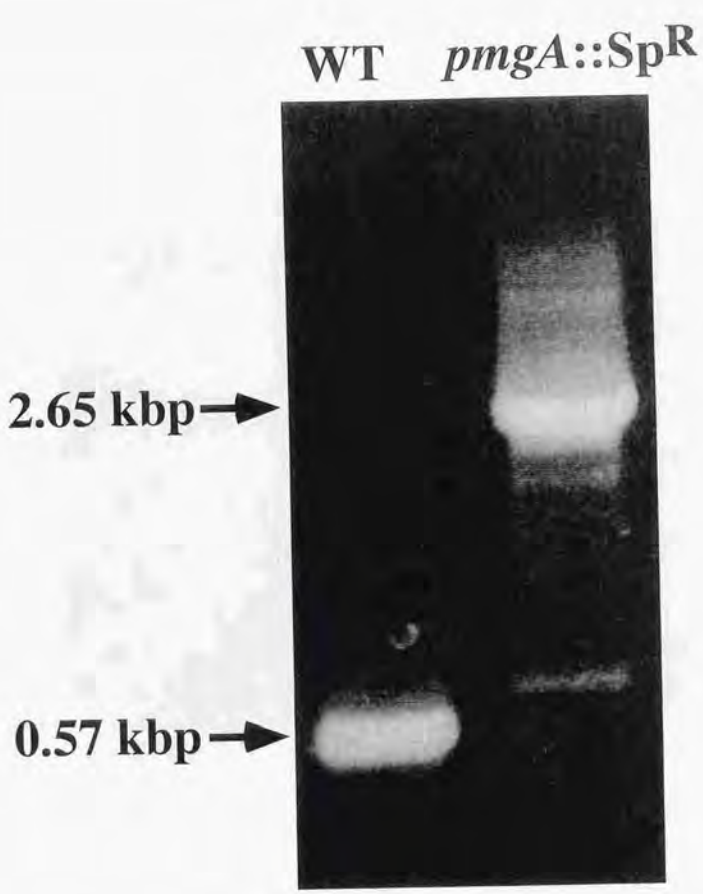



Fig. I-6 *Disruption of pmgA by insertion of a spectinomycin resistance-cartridge.*

Complete segregation was verified by the lack of PCR amplification of a wild-type fragment. Wild type produced 0.57 kbp fragment (left), whereas the disrupted-mutant which contained the spectinomycin resistance-cartridge of 2.08 kbp in length produced 2.65 kbp fragment (right). There was no amplification of 0.57 kbp fragment in the disrupted mutant.

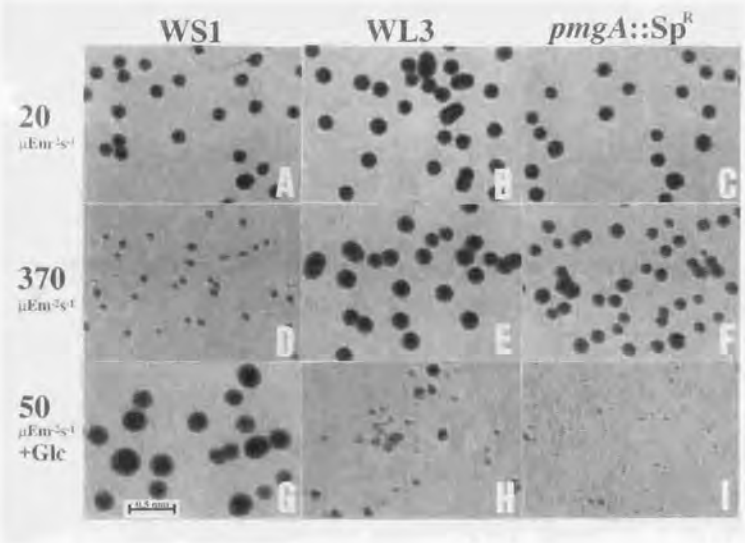



Fig. I-7 *Growth properties of WS, WL and pmgA disrupted mutant under various conditions as demonstrated by colony size.*

WS (A, D, G) , WL (B, E, H) and *pmgA* disrupted mutant (C, F, I) were grown in the light at 20 $\mu Em^{-2}s^{-1}$ for 4 days (A, B, C), 370 $\mu Em^{-2}s^{-1}$ for 3 days (D, E, F) and 50 $\mu Em^{-2}s^{-1}$ with glucose on the plate wrapped with parafilm for 5 days (G, H, I). Note the difference in incubation time for each condition.

# Chapter II

## *pmgA* specifically regulates photosystem stoichiometry in response to high light.

## Introduction

Acclimation to different light regimes is one of the most important and complex responses of photosynthetic organisms to varying environmental conditions. Under different growth light, cyanobacteria and plants regulate accumulation of antenna pigment complexes, photochemical reaction centers, and enzymes for $CO_2$ fixation to optimize utilization of light energy (reviewed by Anderson, 1986; Melis, 1991; Anderson et al., 1995). Under light-limiting conditions, antenna pigments are selectively accumulated to collect light energy efficiently. It is well-known that cyanobacteria increases their antenna size by elongation of the phycobilisome rods and by an increase in the number of phycobilisomes per unit area of thylakoid membrane upon the shift to low light (Knanna et al., 1983; Lönneborg et al. 1985). Higher plants and green algae having chlorophyll *b* as an antenna pigment show a marked decline in chlorophyll *a* to *b* ratio, reflecting accumulation of the light-harvesting chlorophyll *a/b* complex (Leong and Anderson, 1984). On the other hand, under light-saturating conditions, these organisms reduce their antenna size. Moreover, RuBisCO, the rate-limiting enzyme for $CO_2$ fixation, is accumulated to balance with high photochemical activities (Björkman 1981). Expression of enzymes such as catalase and superoxide dismutase is also enhanced to scavenge reactive oxygen species, which are generated by excess light energy (Foyer et al., 1994). The amount of PSII relative to that of PSI, the photosystem stoichiometry, is another target for the regulation in response to light intensity, since photosynthetic electron transport to generate NADPH and ATP is driven by coordination of the two photosystems with

distinct antenna sizes (Melis et al., 1985; Fujita et al., 1987; Fujita et al., 1994). In general, the antenna size of PSII is variable, whereas that of PSI is unchanged under various light conditions. Under low light, the Photosystem stoichiometry is optimized based on their antenna sizes, whereas it must be kept near unity irrespective of the antenna size under high light. Thus, organisms must balance the electron flow between the two photosystems by modulating both antenna complexes and photosystem stoichiometry at different light intensities.

Although a number of reports have provided information on the physiological and biochemical aspects of various light acclimation, very little is known about molecular mechanisms for sensing of light conditions or for modulation of expression and/or assembly of the photosynthetic apparatus (Allen, 1995; Anderson et al., 1995). Here, I show that modulation of photosystem stoichiometry, one of the responses typically observed upon the shift to high light, is specifically supported by *pmgA*, the gene I identified as described in Chapter 1, in the cyanobacterium *Synechocystis* sp. PCC 6803.

## Materials and Methods

*Strains and culture conditions*

A glucose-tolerant wild-type strain of *Synechocystis* sp. PCC 6803 and mutants (WL strain and a disruptant; see Chapter 1) were grown at 32°C in BG-11 medium with 20 mM HEPES-NaOH (pH 7.0) under continuous illumination provided by fluorescent lamps. In time course experiments, fresh media were inoculated at cell density of $OD_{730}$=0.05 with precultures ($OD_{730}$=0.8-0.9) grown under low light (20 $\mu Em^{-2}s^{-1}$) and transferred to the conditions of high light (200 $\mu Em^{-2}s^{-1}$). Unless otherwise stated, cells were grown as 50 ml in volume with test tubes of 3 cm diameter. Photon flux density was measured by a quantum sensor (LI-250; LiCOR, Lincoln, USA). Liquid cultures were bubbled with air containing 1.0% (v/v) $CO_2$. The *pmgA*-disrupted mutant was usually maintained with 20 μg/ml spectinomycin. Cell density was estimated as scattering at 730 nm ($OD_{730}$) with a spectrophotometer UV-160A.

*Absorption and 77 K fluorescence emission spectra*

Absorption spectra of whole cells of wild type and mutants suspended in BG-11 medium were measured at room temperature using a U3500 spectrophotometer (Hitachi, Tokyo, Japan) with an end-on type photomultiplier to minimize the scattering. Content of chlorophyll and phycocyanin was calculated using the equations of Arnon et al. (1974). Low-temperature fluorescence emission spectra at 77 K were recorded using a custom-made apparatus (Sonoike and Terashima, 1994). Cells containing 100 μg chlorophyll/ml in BG-11 medium were placed in a

sample holder. Pigments were excited with blue light produced by passing white light from a 100 W halogen lamp through a filter (CS 4-96; Corning Glass, Corning, N.Y., USA).

*Measurement of electron transfer reactions*

Oxygen evolution and consumption of cells was measured in BG-11 medium with a Clark-type electrode at a chlorophyll concentration of 2.5 μg/ml. The medium was continuously stirred at 25°C and illuminated with saturating actinic light (4000 $\mu Em^{-2}s^{-1}$). Whole cell photosynthetic activity or PSII-mediated electron transfer activity was measured as $O_2$ evolution supported by 2 mM $NaHCO_3$ or 2 mM 2,6-DCBQ, respectively. PSI-mediated electron transfer activity was measured as $O_2$ consumption in the presence of 1 mM ascorbic acid, 5 mM DAD, 2 mM MV, 20 μM DCMU and 1 mM KCN.

*Determination of photosystems*

Thylakoid membranes used for measurements of PSII and PSI were isolated from cells grown in 1000 ml flat vessels. Cells suspended in HN buffer (5 mM HEPES-NaOH, 10 mM NaCl, pH 7.5) were broken with a Mini-Bead Beater (Biospec, Bartlesville, USA) by agitation with zircon beads (100 μm diameter; Biospec) for 3 pulses of 50 s each with 2 min cooling intervals at 0°C. After brief centrifugation to remove the beads, cell debris and thylakoid membranes were collected at 45,000 xg for 20 min with a RP80AT rotor (Hitachi). Pellets were resuspended in HN buffer and sonicated for 3 pulses of 10 s each with 10 s cooling intervals at 0°C to liberate thylakoid membranes from cell debris. After centrifugation at

2,500 xg for 5 min with a RT15A8 (Hitachi), thylakoid-containing supernatants were used to determine P700 and cytochrome *b*559.

PS II content was estimated as a half molar of cytochrome *b*559 as described in Fujita and Murakami (1987) since two cytochrome *b*559 have been believed to be tightly bound to the PSII reaction center complex (Whitmarsh and Ort, 1984). Cytochrome *b*559 was determined from the difference spectrum (520-600 nm) between ascorbate-reduced and hydroquinone-reduced conditions using a U3500 spectrophotometer. A chlorophyll concentration of thylakoid membranes was 80 μg/ml, and a difference absorption coefficient of 21 $mM^{-1}cm^{-1}$ (Garewall and Wasserman, 1974) was used for cytochrome *b*559.

PS I content was estimated as P700 content photooxidized by continuous illumination. Absorbance changes at 703 nm were measured with a spectrophotometer (Model 356; Hitachi) (Terashima et al., 1994). The reaction mixture contained thylakoid membranes at a chlorophyll concentration of 3 μg/ml in 50 mM Tris-HCl (pH 7.5), 10 mM sodium ascorbate, 30 μM TMPD, 10 μM MV and 0.05% dodecylmaltoside. The reduced-minus-oxidized differential absorption coefficient of P700 is known to vary with species (Hiyama and Ke, 1972; Sonoike and Katoh, 1990) or with the preparation used (Sonoike and Katoh, 1988; Sonoike and Katoh, 1989). Thus, I determined the absorption coefficient of P700 in the thylakoid membranes from *Synechocystis* sp. PCC 6803, by measuring oxidation of TMPD coupled with reduction of flash-oxidized P700 basically as described by Hiyama and Ke (1972). Flash-induced absorbance changes in millisecond time scale were measured with a single-beam spectrophotometer (RA-401; Otsuka Electronics, Osaka, Japan) under

aerobic conditions. Absorption changes were measured at 703 nm for P700 and 575 nm for TMPD, and the absorption coefficient of oxidized TMPD at this wavelength was assumed to be 10.7 $mM^{-1}cm^{-1}$ (Hiyama and Ke, 1972). Saturating xenon flashes (half duration time of 5 μs) passed through two band pass filters (CS 4-96 and CS 7-59; Corning) and a dichroic filter (DF-B; Japan Vacuum Optics, Gotenba, Japan) were fired at 0.1 Hz, and signals were monitored with a photomultiplier (R374; Hamamatsu Photonics, Shizuoka, Japan) blocked with two cut off filters (R-69; Toshiba, Tokyo, Japan) for P700 or with an orange cut off filter (O-57; Toshiba) and a dichroic filter (DF-C; Japan Vacuum Optics) for TMPD. The reaction mixture contained thylakoid membranes equivalent to 3 μg/ml chlorophyll, 0.8 mM TMPD, 10 μM DCMU, 1 mM KCN, 0.05% dodecylmaltoside and 50 mM Tris/HCl (pH 7.5). Absorption coefficient of P700 in thylakoid membranes from *Synechocystis* sp. PCC 6803 in the presence of dodecylmaltoside was determined as 71 ± 3 $mM^{-1}cm^{-1}$. This value was significantly greater than that determined for *Synechococcus elongatus* in a similar condition (Sonoike and Katoh, 1988) but close to the value for the PSI preparation from Triton-solubilized thylakoid membranes from *Anabaena variabilis* (Hiyama and Ke, 1972).

*Immunoblot analysis*

Whole cell extracts were treated with LDS before removal of cell debris as described above and were subjected to SDS-polyacrylamide gel electrophoresis. For detection of D2, PsbO and RuBisCO proteins, the extracts were solubilized with 1% LDS, 60 mM dithiothreitol, 60 mM Tris-HCl (pH 8.0) for 10 min at room temperature, while for PsaA/B proteins,

they were solubilized with 5% LDS, 60 mM dithiothreitol, 60 mM Tris-HCl (pH 8.0) for 2 h at room temperature to achieve complete denaturation. SDS gel electrophoresis was done by the procedure of Laemmli (1970) with a gel containing 12.5% acrylamide and 6 M urea for D2, PsbO and RuBisCO or a gradient gel of 16-22% acrylamide with 7.5 M urea for PsaA/B. Samples corresponding to 0.32 x $10^7$, 0.48 x $10^7$, 1.92 x $10^7$ and 1.15 x $10^7$ cells were loaded for the detection of D2, PsbO and RuBisCO and PsaA/B, respectively. Proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Milwaukee, USA). The antiserum against PsaA/B from *Synechococcus elongatus* was kindly provided by Dr. I. Enami (Science University of Tokyo). The antiserum against RuBisCO from spinach was a generous gift from Dr. K. Okada (Tokyo University of Pharmacy and Lifescience). The antisera against spinach were used for detection of D2 and PsbO (Ikeuchi and Inoue, 1987). Reaction with antisera and immunodetection by alkaline phosphatase or peroxidase were performed according to the manufacturer's instructions (Bio-Rad, Richmond, USA).

*Preparation of total RNA*

Cells were collected by brief centrifugation at 4°C and stored in liquid $N_2$. The frozen cells were thawed with 20 mM EDTA and 50 mM Tris-HCl (pH 8.0), and immediately treated with phenol at 75°C for 10 min. Cells were further treated with 0.8%(w/v) SDS at 75°C for 10 min with shaking, and then extracted once with phenol/chloroform and twice with chloroform. After precipitation with ethanol, RNA was solubilized in 8 M guanidine-HCl, 0.1 M sodium acetate, pH 5.2, 5 mM dithiothreitol, 0.5% sodium

lauryl sarcosinate and precipitated with ethanol. Residual DNA in the RNA preparation was removed by digestion with DNase I at 25°C for 2 h. After ethanol precipitation, the amount of RNA was determined by UV absorption at 260 nm.

*RT-PCR*

First strand cDNA was synthesized using 1 μg of total RNA with a kit of RT-PCR High (TOYOBO, Osaka, Japan) in a final volume of 20 μl according to the manufacturer's instructions. The amount of cDNA used as a template was experimentally determined for each set of primers to achieve proportional production of the PCR product. The pairs of oligonucleotide primers, 5'-TGTAAAACGACGGCCAGTCAGCACATTCAGGCCTCC-3' and 5'-CAGGAAACAGCTATGACCGCTTAATTTTCTTGCTGA-3' were used for amplification of a 565 bp fragment of *pmgA* and 5'-AGTTAGGGAGGGAGTTGC-3' and 5'-TAAGCCGGGTTCTGTTCC-3' were used for amplification of a 417 bp fragment of the constitutive RNase P gene, *rnpB*, as a positive control (Frías et al., 1994). After a first denaturation step of 3 min at 93°C, 30 PCR cycles were performed (93°C for 30 sec, 57°C for 2 min, 72°C for 2 min) followed by a final extension step of 10 min at 72°C. As a negative control of RT-PCR, about ten times more RNA without the RT reaction was used as a template for PCR amplification of *rnpB*.

## Results

*Absorption spectra and photosynthetic pigment content*

I isolated a mutant clone with larger colony size (WL strain) compared with smaller colonies of wild type (WS strain) under high light and identified a point mutation in a novel gene, *pmgA*, responsible for the difference in growth rate on agar plates as described in Chapter 1. On the other hand, in liquid culture WL and *pmgA*-disruptant cells showed enhanced pigmentation relative to wild-type cells at 200 $\mu Em^{-2}s^{-1}$ (designated "HL") as shown in Fig. II-1. In addition, mutants showed slightly higher growth rate than wild type. Difference in pigmentation was barely discernible for colonies on agar plates under similar HL conditions or for liquid cultures at 20 $\mu Em^{-2}s^{-1}$ (designated "LL") (data not shown). Figure II-2 shows absorption spectra of cells grown in liquid culture under HL or LL. Absorption spectra were significantly different between the wild type and *pmgA* mutants under HL: the peak of chlorophyll absorption at 678 nm was higher than the peak of phycocyanin absorption at 628 nm in the mutants, while it was lower in wild type (Fig. II-2B). On the other hand, there was no difference in the relative peak heights between wild type and mutants when grown under LL (Fig. II-2A). Compared with HL-grown cells, the content of both pigments increased relative to cell density and to carotenoid absorption ($\approx$ 495 nm) in both cell types under LL.

Figure II-3 shows time course changes in cell density and pigment abundance after HL shift in the batch culture defined in Materials and Methods section. Cultures were inoculated with cells grown to late log phase ($OD_{730}$= 0.8-0.9) under LL and then transferred to the HL condition.

Data at time 0, representing LL-grown cells, showed no significant differences between wild type and *pmgA* mutants. Upon transfer to HL, the content of both chlorophyll (Fig. II-3B) and phycocyanin (Fig. II-3C) on a per cell basis showed changes with three different phases: (1) drastically reduced to about 2/3 within 3 h, (2) further decreased but at a lesser rate until 12 h, (3) gradually recovered, while cells continued to grow logarithmically in all phases (Fig. II-3A). Notably, the chlorophyll content was significantly greater in *pmgA* mutants than in wild type after 9 h, whereas the phycocyanin content was not much different between the strains throughout the batch culture. The cellular content of both pigments was almost recovered to the initial level after 30 h under HL conditions probably due to the self-shading effect at high cell density. Taking into account that cells were dividing logarithmically, accumulation of pigments, expressed as per ml of culture volume, was shown on a log scale in Figs. II-3D and 3E. Accumulation of both pigments paused during the initial 3 h (phase 1). The pigment accumulation re-started at low rate from 3 to 12 h (phase 2) and accelerated after 12 h (phase 3). Clearly, the *pmgA* mutants differed from wild type in their chlorophyll accumulation during phase 2 (Fig. II-3D), leading to a higher cellular chlorophyll content in phase 3 (Fig. II-3B). On the other hand, accumulation of phycocyanin did not differ between wild type and the mutants (Fig. II-3E), although its time course was similar to that of chlorophyll. In short, loss of *pmgA* function seems to abolish the specific retardation of chlorophyll synthesis at phase 2 (3 to 12 h) in response to high light. However, the initial suppression of chlorophyll synthesis, the recovery of chlorophyll synthesis during phase 3,

and phycocyanin synthesis does not seem to be affected by the *pmgA* mutation.

*Chlorophyll-fluorescence spectra and content of photosystems*

Differences in chlorophyll content are assumed to reflect changes in photosystems, since cyanobacteria have no apparent chlorophyll-binding antenna proteins. It is widely accepted that chlorophylls of PSII emit fluorescence around 685 nm and 695 nm, while chlorophylls of PSI emit at 720-730 nm at 77 K (Murata et al., 1966). Thus, as shown in Fig. II-4, I investigated the chlorophyll-fluorescence emission spectra of cells at 77 K to know whether photosystem stoichiometry is different between wild type and *pmgA*-mutants or not. When the spectra were normalized at the peaks of PSI fluorescence, it is notable that the peak at 695 nm originating from PSII is about 1.5-fold higher in wild-type cells grown under HL than under LL (Fig. II-4A). On the other hand, the 695 nm peak was virtually unchanged in *pmgA* mutants (Figs. II-4B and 4C). This strongly suggests that the ratio of PSII to PSI increased in wild type in response to HL, whereas it remained unchanged in the mutants. Since the fluorescence ratio (F695/F725) is a good index of the ratio of PSII/PSI, the ratio was plotted in the same experimental conditions as in Fig. II-2 (Fig. II-5). Clearly, the ratio gradually increased in the wild type 12 h after the shift to HL, reaching the maximum (about 1.5 fold of the initial) around 18 to 24 h, whereas the ratio did not change in the *pmgA* mutants. These differences in time course changes of the F695/F725 ratio between wild type and *pmgA* mutants appear to reflect the difference in chlorophyll accumulation (Fig. II-3D).

To confirm the results of fluorescence measurement, I directly determined the photosystem stoichiometry by measuring cytochrome *b*559 and P700 in thylakoid membranes isolated from LL-and HL-grown cells. Cytochrome *b*559 has been known to be tightly associated with PSII reaction center in thylakoid membranes as a molar ratio of 2:1 (Whitmarsh and Ort, 1984), while P700, a photoactive pigment of PSI reaction center, was used to determine PSI content (Hiyama and Ke, 1972). Table II-1 shows that the PSII content on a per cell basis decreased to about 74% in wild type during the first 13 h after the shift to HL conditions, while the PSI content markedly decreased to about 40% of the initial value. As a result, the ratio of PSII/PSI increased from 0.48 for LL-grown cells to 0.81 for HL-grown wild-type cells. Clearly, *pmgA* mutants did not show this change of photosystem stoichiometry, mainly due to the absence of the marked loss of the PSI content in HL cells. Wild-type cells showed a slight recovery of their photosystem content and stoichiometry after 22 h. Consistently, low temperature fluorescence ratio (F695/F725) of the wild-type thylakoids was at the maximum level at 13 h and slightly lower at 22 h, whereas the ratio of the mutant thylakoids was not much changed (Fig. II-6). These fluorescence changes seemed to occur slightly earlier than those observed in Fig. II-5. This may be due to higher self-shading in the larger culture volume required for the thylakoid isolation. From the data in Table II-1 and content of phycocyanin, the ratio of phycocyanin to PSII, which represents antenna size of PSII, was also calculated (Table II-2). It dropped to about 80% not only in wild type but also in *pmgA* mutants during the first 13 h after the shift to HL conditions. Thus, it was concluded that *pmgA* is essential for the modulation of the PSII/PSI ratio as acclimation to

high light but not for the adjustment of antenna size of PSII. Probably, the photosystem stoichiometry was modulated by accumulation of PSI complex.

*Electron transfer activities under different light intensities*

I pursued the relationship between the two phenotypic features of *pmgA* mutants, the lack of modulation of the photosystem stoichiometry, and the enhanced growth under photoautotrophic conditions, by measuring photosynthetic activities (Table II-3). Under LL conditions, there were no significant differences in whole cell photosynthetic activity (from water to $CO_2$) between wild type and *pmgA* mutants. This is consistent with my previous observation that the growth rate of *pmgA* mutants was comparable to that of wild type under LL (Figs. I-2,7). Strangely, PSI activity of the mutants (from reduced DAD to MV) was much lower than that of wild type, while the P700 content was not significantly different. Since DAD indirectly donates electrons to P700, the difference in the PSI activity may be affected by other unknown factors. When cells were grown under HL conditions, the whole photosynthetic activity of the mutants was much greater than that of wild type. This seems to account for the observation that the *pmgA* mutants grew slightly better than wild type under HL (Fig. II-3A). The PSI activity was higher in *pmgA* mutants than wild type, reflecting higher PSI content. The PSII activity was also higher in *pmgA* mutants, despite that the PSII content was not affected by the mutation (Table II-1). Possible reason will be discussed in Discussion section in terms of photoinhibition.

It is also of note that the ratio of the whole photosynthetic activity to PSII or PSI activity was much higher in HL-grown cells than in LL-grown

cells, regardless of functionality of *pmgA*. This suggests that enzyme(s) for $CO_2$ fixation were up-regulated under HL, in contrast to the down regulation of chlorophyll content. Both regulations are typical response of photosynthetic organisms to high light conditions.

*Western blotting analysis*

In order to confirm the measurements of photosystem content and activities, proteins of photosystems and RuBisCO were probed with specific antibodies as shown in Fig. II-7. Clearly, the cellular content of the D2 protein of PSII reaction centers, PsbO protein of PSII oxygen evolving complexes, and PsaA/B proteins of PSI reaction centers were down-regulated under HL, whereas the content of RuBisCO large subunit was up-regulated. It is of note that only PsaA/B proteins under HL were specifically down regulated to a greater extent in wild type than in *pmgA* mutants. These results appeared to agree with the effects of HL on photosystem content and the whole photosynthetic activity and further confirm the role of *pmgA* in regulation of the PSII/PSI ratio.

*RT-PCR of pmgA mRNA*

Although I have shown that *pmgA* is essential for modulation of photosystem stoichiometry, molecular processes mediated by *pmgA* are still unknown. To learn more about the role of *pmgA*, I investigated expression of *pmgA* in wild type by RT-PCR as shown in Fig. II-8. In contrast to relatively constant amplification of the constitutive RNase P gene (*rnpB*) (Frías et al., 1994), production of a *pmgA* fragment was largely dependent on the light condition. When cells were grown under LL, cDNA for *pmgA*

was barely detectable as can be seen at time 0. After 4 h of HL illumination, cDNA for *pmgA* increased several fold and was then maintained at the high level for up to 16 h. It is also of note that the amount of cDNA sufficient for PCR amplification was more than 10,000 times higher for *pmgA* than *rnpB*, suggesting that expression of *pmgA* is very low even under inducible conditions. We confirmed practically no DNA contamination in the RNA preparations before the reverse transcriptase reactions, as shown in the negative control experiments. These demonstrate that HL-induced expression of *pmgA* preceded the *pmgA*-dependent changes in both chlorophyll content and PSII/PSI ratio in wild type.

## Discussion

I have demonstrated that functional *pmgA* is specifically involved in acclimation to high light by modulating photosystem stoichiometry and, partly, chlorophyll biosynthesis. In my experimental conditions with *Synechocystis* sp. PCC 6803, I could observe many changes due to high light acclimation: (1) decrease of cellular pigment content, (2) decrease of photochemical activities (on a per cell basis) and (3) decrease of antenna size of PSII, (4) increase of the PSII to PSI ratios, (5) increase of RubisCO and (6) increase of maximum photosynthetic rate on a per cell basis. These responses, possibly resulting from high light-induced modulation of accumulation of pigments and proteins for the photosynthetic apparatus, have been widely recognized in cyanobacteria, algae, and higher plants (Kawamura et al., 1979; Vierling and Alberte, 1980; Björkman, 1981; Raps et al., 1983; Zevenboom and Mur, 1984; Anderson et al., 1988). In Results section, I showed that *pmgA* is specifically responsible for the slow recovery of chlorophyll accumulation during phase 2 after the shift from LL to HL (Fig. II-3D) and for an increase in the PSII to PSI ratio (Figs. II-4, II-5 and Table II-1). Importantly, *pmgA* is not directly involved in other responses. This is the first gene to be identified as a regulatory factor for high-light acclimation in plants and cyanobacteria. Since modulation of photosystem stoichiometry under different growth irradiances has been well documented in various cyanobacteria, algae and higher plants (Kawamura et al., 1979; Falkowski et al., 1981; Leong and Anderson, 1984; Neale and Melis, 1986; Wild et al., 1986; Anderson et al., 1988; Smith and Melis, 1988; Murakami and Fujita, 1991; Yokoyama et al., 1991), it would be very

interesting to survey other cyanobacteria and/or plants for a *pmgA* homolog.

The novel phenotype that photoautotrophic growth of *pmgA* mutants on agar plates was much better than wild type under high light conditions (Figs. I-2,7) was totally unexpected. I was able to account for it, at least in part, based on the measurement of photosynthetic activities. Namely, *pmgA* mutants showed elevated activities in PSII, PSI and whole photosynthesis when grown under high light conditions (Table II-3). The higher activity of PSI is consistent with the results of P700 measurements (Table II-1) and immunoblotting of PSI reaction center PsaA/B proteins (Fig. II-7). On the other hand, the PSII activity of the mutants was also higher than wild type (Table II-3), although the cellular content of cytochrome *b*559 and reaction center D2 protein were not much different between the mutant and wild-type cells (Table II-1 and Fig. II-7). This might be explained by selective photoinhibition of PSII in wild type under HL growth conditions. Photoinhibition of PSII is known to be caused by accumulation of reduced quinone at the primary acceptor $Q_A$ site (Aro et al., 1993). The higher activity of PSI in the mutants is supposed to extract more electrons from PSII, resulting in less inhibition of PSII. However, if the amount of RuBisCO protein was not affected by the mutation in *pmgA* as was suggested by Western blotting (Fig. II-7), it would be difficult to expect the higher photosynthetic activity in the mutants than in wild type. The RuBisCO activity is widely believed to be rate-limiting in the whole photosynthetic pathway in many organisms. We may have to consider the possibility that the higher activities of PSII and PSI in the mutants may lead to more activation of the RuBisCO activity and/or slightly more induction

of RuBisCO protein. Anyway, mutation in *pmgA* appears to be directly or indirectly linked to the higher photosynthetic activity and the higher growth rate.

How does *pmgA* work on the accumulation of chlorophyll and modulation of photosystem stoichiometry? Our data of photosystem content suggest that photosystem stoichiometry was mainly modulated by accumulation of PSI. It is consistent with studies on light acclimation in cyanobacteria which demonstrated that cellular PSI content is more variable than PSII during the adjustment of photosystem stoichiometry (Kawamura et al., 1979; Murakami and Fujita, 1991). However, it remains to be determined whether chlorophyll accumulation or PSI accumulation is the primary target of the *pmgA*-mediated acclimation to high light. The retardation of chlorophyll accumulation (Fig. II-3D) seemed to precede the increase of the F695/F725 ratio (Fig. II-5). However, it would be rather difficult to imagine a mechanism whereby chlorophyll biosynthesis selectively regulates assembly of the PSI complex. It should be noted that mRNA level of *pmgA* was elevated several fold in the initial 4 h of HL illumination (Fig. II-8). This suggests that transcription of *pmgA* responds to HL very early in the acclimative process. The deduced product of *pmgA* has limited motifs resembling G1 and G2 boxes of histidine kinase in bacterial two-component regulatory systems, although the conserved histidine residue is not present in *pmgA* (Fig. I-5). G1 and G2 boxes of histidine kinases are hypothesized as nucleotide-binding sites (Egger et al., 1997). These observations, coupled with the fact that the level of *pmgA* is very low even after HL induction, suggest that the *pmgA* product acts as a regulatory factor on an early step of the high light acclimation.

So far, two genes have been documented as being specifically involved in accumulation of PSI complexes but not PSII (Wilde et al., 1995; Bartsevich and Pakrasi, 1997; Boudreau et al., 1997). Disruption of a chloroplast open reading frame, *ycf4*, and a *Synechocystis* homolog, *orf184*, induced a significant decline in PSI content per chlorophyll. As a result, the PSII to PSI ratio was elevated about 3-fold in the mutant compared with wild-type *Synechocystis* (Wilde et al., 1995), while there was almost no accumulation of PSI complex in the *Chlamydomonas* mutant (Boudreau et al., 1997). The second gene, *btpA*, seems to regulate a post-transcriptional process to affect biogenesis of the PSI complex in *Synechocystis* (Bartsevich and Pakrasi, 1997). A disruption mutant of *btpA* had only 10 - 15% of PSI reaction center proteins compared with wild type while the PSII content remained unaffected. These different genes may regulate accumulation of PSI at a step of translation, assembly or turnover of the PSI complex, although no relevant data were presented to indicate their involvement in physiological adjustment of photosystem stoichiometry under varying environment conditions. To our knowledge, *pmgA* is the first gene shown to be involved in the regulation of PSII/PSI ratio under physiological conditions.

Table II-1 *Content of photosystems in wild type and pmgA mutants.*

| Strain | Time | PSII | PSI | PSII/PSI |
|---|---|---|---|---|
| | | ($10^{-19}$mols/cell) | | |
| WS | 0 h | 2.01±0.19 | 4.21±0.13 | 0.48±0.06 |
| (wild type) | 13 h | 1.48±0.23 | 1.68±0.11 | 0.81±0.08 |
| | 22 h | 1.55±0.16 | 2.13±0.19 | 0.73±0.07 |
| WL | 0 h | 2.17±0.38 | 4.27±0.08 | 0.51±0.08 |
| | 13 h | 1.57±0.05 | 2.67±0.24 | 0.60±0.07 |
| | 22 h | 1.85±0.04 | 3.49±0.32 | 0.53±0.06 |
| *pmgA*::Sp$^R$ | 0 h | 2.10±0.32 | 4.16±0.21 | 0.50±0.07 |
| | 13 h | 1.47±0.09 | 2.51±0.18 | 0.59±0.04 |
| | 22 h | 1.79±0.08 | 4.31±0.52 | 0.42±0.04 |

Numbers of PSI amd PSII on a per cell basis were calculated from P700 and a half of cytochrome $b_{559}$, respectively. Data are the means ±SE for at least three separate experiments.

The culture was inoculated with LL (20 $\mu Em^{-2}s^{-1}$)-grown cells and transferred to HL (200 $\mu Em^{-2}s^{-1}$) at time 0. Conditions for the culture were the same as in Fig. II-3, except for larger culture volume.

Table II-2 *Antenna size of Photosystem II.*

| | Antenna size (pg Phycocyanin / Photosystem II) | | |
|---|---|---|---|
| Time | WS | WL | *pmgA*::Sp$^R$ |
| 0 h | 0.238±0.020 | 0.224±0.030 | 0.217±0.030 |
| 13 h | 0.187±0.009 | 0.187±0.010 | 0.184±0.008 |
| 22 h | 0.204±0.016 | 0.218±0.017 | 0.211±0.025 |

Antenna size of PS II was calculated from the same samples which were used for the determination of the photosystem content.

Table II-3 *Photosynthetic activities of cells.*

20 $\mu Em^{-2}s^{-1}$ [a]

| Reaction | Activities ($\mu molO_2$evolved/$10^9$cells/h) | | |
|---|---|---|---|
| | WS | WL | *pmgA*::Sp$^R$ |
| $H_2O$ to $HCO_3^-$ | 6.60±0.51 | 6.49±0.50 | 6.53±0.77 |
| PSII ($H_2O$ to 2,6-DCBQ) | 21.72±0.30 | 22.73±0.51 | 22.27±0.69 |
| PSI (DAD/Asc to MV) | -19.59±3.31 | -13.41±2.82 | -14.43±2.89 |

200 $\mu Em^{-2}s^{-1}$ [b]

| Reaction | Activities ($\mu molO_2$evolved/$10^9$cells/h) | | |
|---|---|---|---|
| | WS | WL | *pmgA*::Sp$^R$ |
| $H_2O$ to $HCO_3^-$ | 8.87±0.16 | 12.41±1.06 | 11.35±0.55 |
| PSII ($H_2O$ to 2,6-DCBQ) | 9.24±0.33 | 13.56±1.41 | 12.67±0.83 |
| PSI (DAD/Asc to MV) | -6.02±0.74 | -11.24±3.53 | -11.41±2.04 |

All values represent the means±SE for at least three separate experiments.

[a] Activities of cells grown under LL ( 20 $\mu Em^{-2}s^{-1}$) for 2 days.

[b] Activities of cells grown under HL ( 200 $\mu Em^{-2}s^{-1}$) for 18 h.


Fig. II-1 *Liquid culture of wild type (WS) and pmgA mutants (WL and the disruptant "pmgA::Sp$^R$") at log phase under HL conditions (200 $\mu Em^{-2}s^{-1}$).*

$OD_{730}$ of WS, WL and pmgA-disruptant were 0.56, 0.73 and 0.66, respectively.

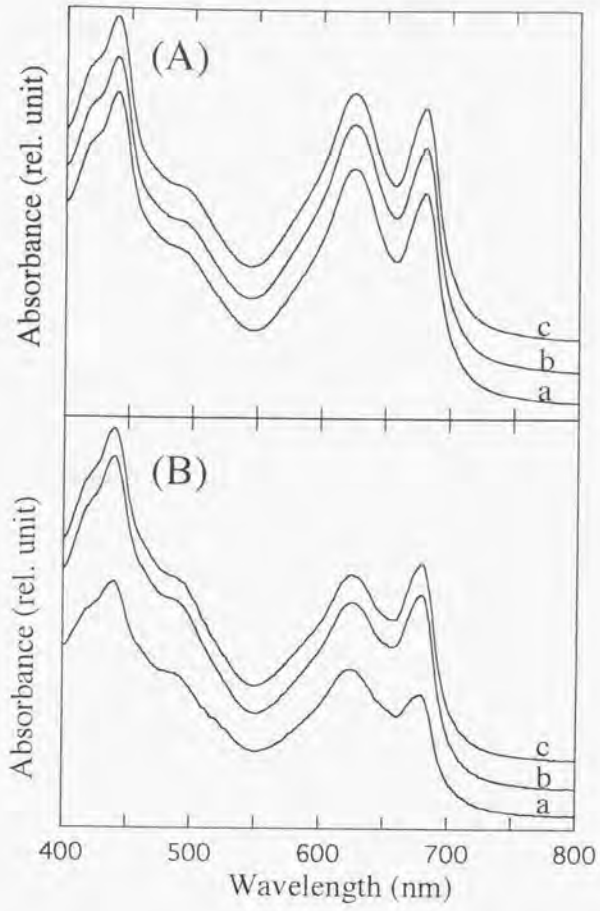



Fig. II-2 *Absorption spectra of cells grown at different light intensities.*

(A) Absorption spectra under LL (20 $\mu Em^{-2}s^{-1}$).
(B) Absorption spectra under HL (200 $\mu Em^{-2}s^{-1}$).
The spectra of WS (a), WL (b) and *pmgA*-disruptant cells (c) are normalized at $OD_{730}$.

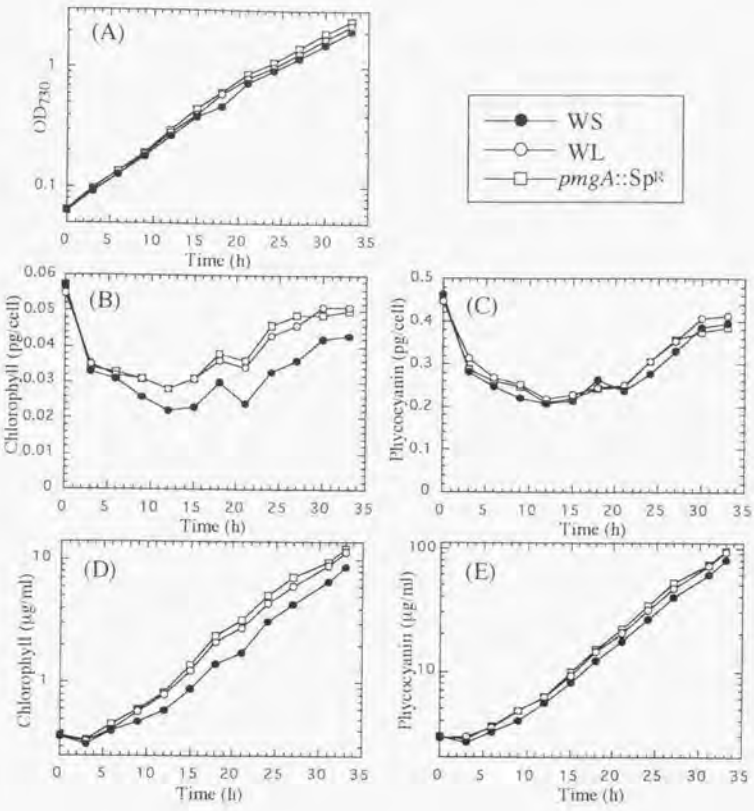



Fig. II-3 *Growth curve and changes in the pigment content in the course of a batch culture under HL (200 μEm-2s-1).*

(A) Cell density expressed as $OD_{730}$.
(B) Chlorophyll content expressed as per cell basis.
(C) Phycocyanin content expressed as per cell basis.
(D) Chlorophyll accumulation expressed as per ml of culture volume.
(E) Phycocyanin accumulation expressed as per ml of culture volume.
At time 0, batch culture was inoculated with LL (20 μEm-2s-1)-grown cells.

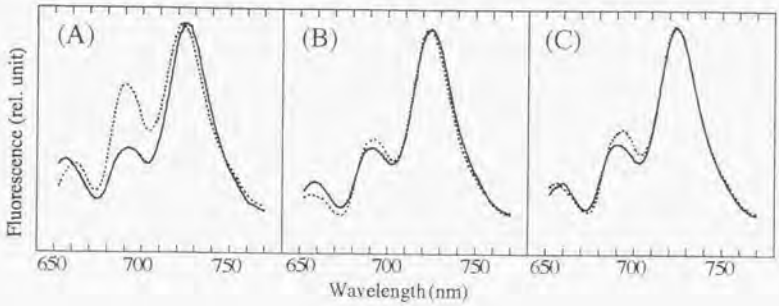



Fig. II-4 *Low temperature (77 K) fluorescence emission spectra of cells.*

(A) Fluorescence emission spectra at 77 K of WS.
(B) Fluorescence emission spectra at 77 K of WL.
(C) Fluorescence emission spectra at 77 K of *pmgA*-disruptant.
Spectra of cells grown under LL (20 $\mu Em^{-2}s^{-1}$) for 2 days(solid line) and cells grown under HL (200 $\mu Em^{-2}s^{-1}$) for 20 h (dashed line) were normalized at the 725 nm peak of PSI.

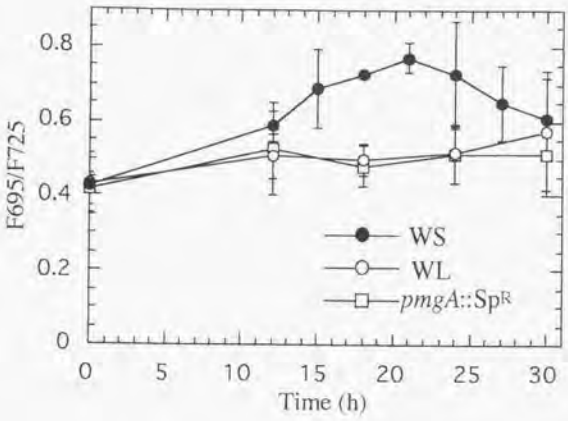



Fig. II-5 *Time course of the change in the ratio of F695/F725 in the course of a batch culture under HL (200 $\mu Em^{-2}s^{-1}$).*

Conditions for the culture were the same as in Fig. II-3. Data are the means ± SE for at least three separate experiments.

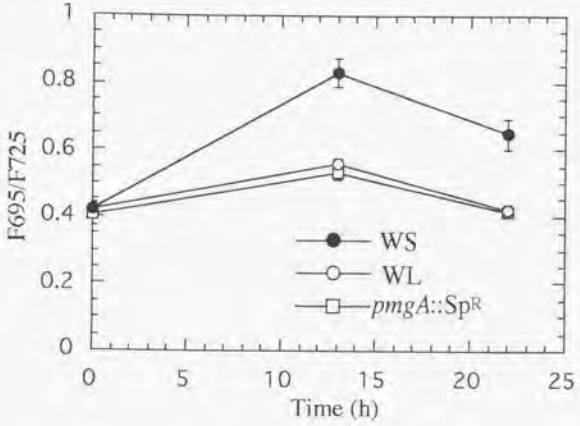



Fig. II-6 *Time course of the changes in the ratio of F695/F725 of the same samples as in Table II-1.*

The changes in the ratio of F695/F725 of the same samples, which were used for the determination of the photosystem content were examined. Data are the means ± SE for at least three separate experiments.

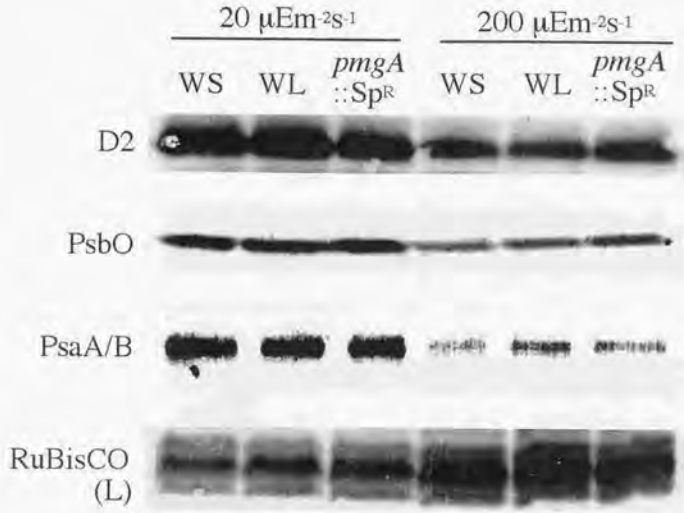



Fig. II-7 *Immunoblotting of polypeptides of wild type and pmgA mutants.*

Total cell extracts of LL (20 $\mu Em^{-2}s^{-1}$)- or HL (200 $\mu Em^{-2}s^{-1}$)-grown cells of WS, WL and pmgA-disruptant were separated by SDS-PAGE, electrotransferred and challenged with anti-D2, anti-PsbO, anti-PsaA/B or anti-RuBisCO. Proteins extracted from the same number of cells were loaded for each antibody as described in Materials and Methods section.

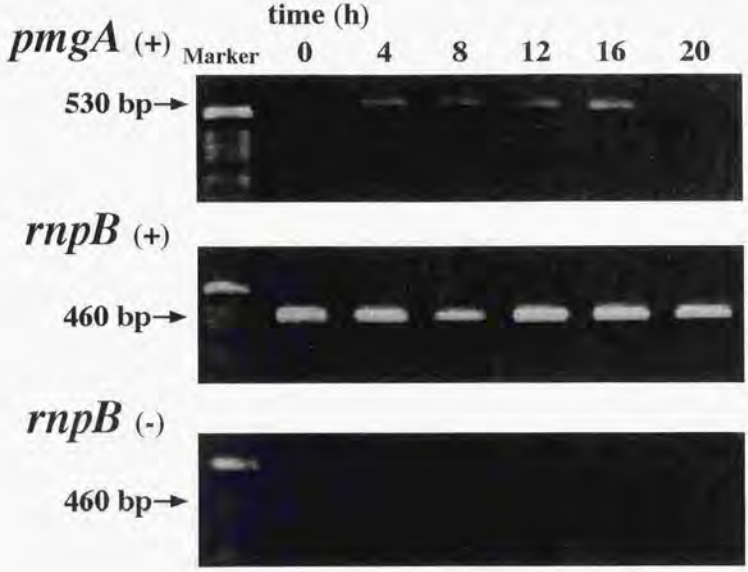



Fig. II-8 *Expression of pmgA in wild type revealed by RT-PCR.*

Upper and middle/lower panels show PCR with primers specific to *pmgA* and *rnpB*, respectively. (+) indicates use of reverse-transcribed RNA from wild-type cells at HL (200 $\mu Em^{-2}s^{-1}$) as a template of PCR. (-) indicates use of RNA before reverse-transcription as a template. The culture for RNA isolation was inoculated with LL-grown cells and transferred to the HL (200 $\mu Em^{-2}s^{-1}$) at time 0.

## Chapter III

# Physiological significance of *pmgA*-mediated acclimative response dissected from evolutionary point of view.

## Introduction

*pmgA* was initially identified as an essential factor required to support photomixotrophic growth with light and glucose. Moreover, in Chapter 2, I showed that *pmgA* is involved in regulation of photosystem stoichiometry under high light conditions. From these observations, *pmgA* seems to play an important role in cyanobacterial acclimation process(es). However, WL, which had non-functional *pmgA*, could grow better than wild type under photoautotrophic conditions and occupied a considerable part in "wild-type" culture in our laboratory as shown in Chapter 1. In general, wild type have acquired the optimal genotype for reproduction and survival in the long-term evolution and thus it is assumed that mutants must have some disadvantages. Why, then, could WL increase in the "wild-type" culture? There are several possible answers to the question. First, WL cells may have increased by random drift and be foredoomed to extinction sooner or later. Second, WL may be more advanced genotype than wild type with respect to photoautotrophic growth. And third, WL phenotype may be superior to wild type only in very limited conditions such as those for maintenance of "wild-type" cultures in the laboratory. To examine which answer is correct is very intriguing and important since it gives us some insights into the significance of *pmgA*-mediated acclimation process from the physiological point of view.

Studies on the polymorphism of *pmgA* is also important from a genetic point of view. Since nucleotide sequencing is now becoming extremely productive in scale, genetic information is increasingly accumulating in databases year by year. Under such conditions, we often

see discrepancies between nucleotide sequences of the same gene not only from different strains but also from the same strains from different laboratory. One typical example is the entire genome sequence of *Escherichia. coli* determined by two groups independently: there found inversion of a nucleotide sequence more than 1 Mb in length and locations of transposons are quite different between the two strains (http://susi.bio.uni-giessen.de/ecdc.htm/; http://genome4.aist-nara.ac.jp/). It seems that for the last several decades the common ancestor has differentiated either inevitably or spontaneously. However, in most cases including the case of *Escherichia. coli*, we have almost no idea about evolutionary significance of variation in sequences. In this sense, the *pmgA* polymorphism may be a very rare case that can show us the progress of evolution in real time and allow us to reproduce the evolution under various environmental conditions.

A direct sequencing is a simple and quantitative tool to detect alleles with point mutation and has been vigorously performed in areas such as studies on hereditary disease. This method can determine a content of heterozygote easily by detection of heterogeneous base signals at the same position in DNA sequencing. In this chapter, using this method, I first examined change of population of WL genotype in the past cultures of our "wild type". Next, I performed mixed culture experiments of WS (wild type) and WL under various growth conditions, and evaluated both advantage and disadvantage of the absence of *pmgA*.

## Materials and Methods

*Strains and culture conditions*

A glucose-tolerant wild-type strain of *Synechocystis* sp. PCC 6803 (WS) and a mutant (WL) were grown at 32°C in BG-11 medium (Stanier et al., 1971) with 20 mM HEPES-NaOH (pH 7.0) under continuous illumination provided by fluorescent lamps. Cells were grown in 50 ml volume with test tubes of 3 cm diameter. Photon flux density was measured by a quantum sensor (LI-250). Liquid cultures were bubbled with air containing 1.0% (v/v) $CO_2$. To support photomixotrophic growth, glucose was added to a concentration of 5 mM. Cell density was estimated as scattering at 730 nm ($OD_{730}$) with a spectrophotometer UV-160A. Solid medium of BG11 was supplemented with 5 mM TES-KOH (pH 8.2), 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate.

*Direct sequencing analysis*

For direct sequencing, PCR amplification followed by cycle sequencing with dye-labeled primers was performed according to the manufacturer's instructions (Applied Biosystems). PCR was carried out with genomic DNA using Primer 3 and Primer 4 that had nucleotide sequences of -21M13 and M13 reverse primers at their 5'-ends, respectively:

Primer 3 (5'-TGTAAAACGACGGCCAGTCAGCACATTCAGGCCTCC-3'), and Primer 4 (5'-CAGGAAACAGCTATGACCGCTTAATT TTCTTGCTGA-3'). PCR products were diluted fivefold with water and subjected to the second cycle sequencing reaction with dye-labeled -21M13

or M13 reverse primers. Sequencing with dye-labeled primers using a Model 373S sequencer gave a relatively uniform signal intensity with slight fluctuation depending on base species.

## Results

*Preparing the standard curve*

Direct sequencing does not include a cloning step, so that the signal ratio of T to C at position 193 of *pmgA* is expected to directly reflect the ratio of WL to WS cells. Moreover, it provides information about the total cells, irrespective of whether they are alive or non-alive at the time of sampling. Thus, it is a very effective method to estimate growth rate of two strains in a mixed culture such as our "wild-type" culture containing WS and WL strains. Before starting, I evaluated the method as quantitative analysis. A series of mixture containing WS and WL genomic DNA was used as standard template for the direct sequencing. The ratio of signal height at the mutation site showed very good correlation with the ratio of the mixed genomes (Fig. III-1). However, 10% of heterogeneity could not be always distinguished from background noise and, therefore, is close to the detection limit of this method.

*WL cells had expelled WS from "wild type"*

First, I tried to answer the question when and how WL cells evolved in our laboratory. We have been maintaining the Williams' glucose-tolerant strain as "wild type" mainly under photoautotrophic conditions at about 50 $\mu Em^{-2}s^{-1}$ from 1988 and often preserved the cultures at -80°C. Thus, I could examine the composition of genome of past cultures. Direct sequencing revealed that cells in 1991 and 1995 showed 100% WS (wild type) genotype (Fig. III-2). Notably, genomic DNA isolated from cells in March 1996 gave a mixed signal, which corresponded to 40% WL based on

our standard curve. Surprisingly, cells in June 1996 showed 100% WL. I also examined the photoautotrophic growth on minimal plates in high light intensity (370 $\mu Em^{-2}s^{-1}$). Consistently, cells in April 1991 and cells in June 1996 gave 100% small and large colonies at high light, respectively. However, cells in June 1995 gave ca. 12% large colonies, despite that the direct sequencing indicated virtually 100% WS. I picked up more than 10 large colonies from the cells in June 1995, grew them for several days on a plate in medium light (50 $\mu Em^{-2}s^{-1}$), and then isolated genomic DNA. Direct sequencing revealed 100% WL for those large colonies, indicating that the cells in June 1995 contained a small portion of WL below detection limit of the sequencing. This observation well coincides with the fact that I noticed the polymorphism of wild-type culture first in August 1995. Unfortunately, I could not examine the phenotype of cells in March 1996, as I preserved only DNA. In conclusion, it took about one year for WL cells to expel WS cells from "wild-type" culture which has been maintained in our laboratory. It is also of note that the original PCC strain carried the WS-type *pmgA*, although it was difficult to verify its phenotype by examining the colony size because of motility.

*Mixed culture experiments under different growth conditions*

In order to know whether the domination of WL is reproducible or not, I attempted to evaluate the growth rate of WL and WS cells in mixed culture. The ratio of WL to WS genotype was again determined by the direct sequencing. Under photoautotrophic conditions, three consecutive batch cultures resulted in gradual increase of the WL genotype at 200 $\mu Em^{-2}s^{-1}$ (Fig. III-3). On the other hand, the content of WL genotype drastically

dropped during the first photomixotrophic growth. After the second culture with glucose, the WL genotype could be no more detected by the direct sequencing. Thus, I confirmed that WL type of *pmgA* has advantage in photoautotrophic growth, while has serious disadvantage in the photomixotrophic growth.

*Mixed culture experiments at various light intensities*

Next, I examined whether or not WL cells are absolutely superior to WS cells under photoautotrophic conditions under any light conditions. Since expression of *pmgA* is normally induced under high light and *pmgA* is involved in high-light acclimation as shown in Chapter 2, *pmgA* may be needed under special conditions of high light. When cultures were transferred to new medium every two days, WL became dominant even under 400 $\mu Em^{-2}s^{-1}$ as shown in Fig. III-4. However, when cells were kept diluted under high light conditions by frequent transfer, WL suddenly disappeared from the mixed culture after the third culture under 200-300 $\mu Em^{-2}s^{-1}$. This disappearance of WL indicates that the *pmgA* mutation is fatal under prolonged stress conditions of high light, suggesting that modulation of the PSII/PSI ratio has been selected as an adaptation to high light in evolution.

## Discussion

In this chapter, I examined the significance of having a functional *pmgA* from the evolutionary point of view. I showed that WL cells spontaneously appeared in wild-type culture a few years ago and completely drove the original WS cells away from the culture. The replacement did not occur by chance. However, observations that WL cells could not tolerate photomixotrophic conditions or prolonged high-light stress indicate that WL is not a more advanced genotype than WS in natural environment which usually fluctuates. The condition where WL can dominate must be limited: a batch culture with inorganic medium under normal to high light. This is almost the same condition as we maintained cultures in the laboratory. It can be concluded that WL cells did evolve to adapt specifically to our laboratory conditions.

As shown in Fig. III-6, *Synechocystis* sp. PCC 6803 has greatly changed its phenotype since isolated from a lake of California. Initially, it was sensitive to high light or photomixotrophic conditions and also active in motility (Rippka et al., 1979). From these cells, Dr. Williams obtained so called glucose-tolerant strain which was tolerant to high light and photomixotrophic conditions and lost motility (Williams, 1988). Recently, the gene which may be responsible for the difference between the original and the glucose-tolerant strain was identified in our laboratory (A.Kamei, unpublished result). The 154 bp which contained 5' portion of a putative gene, *slr2031*, was lost in the glucose-tolerant strain (Katou et al., 1995). When *slr2031* of the original strain was disrupted, the mutant showed resistance against high light, higher efficiency of transformation and lack of

motility like the glucose-tolerant strain. Here, I discovered that WL cells evolved from the glucose-tolerant strain. This strain is more adapted to photoautotrophic growth under laboratory conditions than the glucose-tolerant strain. From these observations, it can be assumed that *Synechocystis* sp. PCC 6803 is still evolving gradually in order to adapt to laboratory conditions.

Such a "microevolution in the laboratory" must not be a rare phenomenon. In the case of organisms which have shorter generation time and smaller copy numbers of genome than *Synechocystis* sp. PCC 6803, it may proceed more drastically. This can be exemplified by *Escherichia coli* as described in Introduction section, although the microevolution in *Escherichia coli* has not yet been analyzed from the functional point of view. In the course of culture maintenance in laboratories, organisms may tend to discard the capacity to acclimate to various natural environment, and choose the advantage for efficient growth under consistent conditions. We must always pay attention to such a possibility whenever we study on acclimation to natural environments.

Finally, what is the original function of *pmgA*-mediated acclimation under the natural environment? In this chapter, It was suggested that functional *pmgA* is indispensable to survival under photomixotrophic conditions and prolonged stress condition of high light. However, the role of regulation of photosystem stoichiometry under high light conditions and relationship between high light and photomixotrophic conditions are still unclear. I will discuss about these problems in General Discussion section.

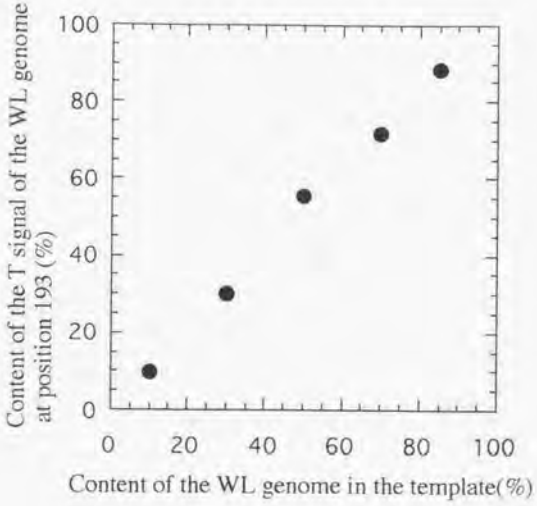



Fig. III-1 *Standard curve of the WL signal relative to the content of the WL genome in the template of the direct sequencing.*

Content of the T signal of the WL genome (ordinate) was calculated based on the peak height of the T and C signals at position 193 of *pmgA*.

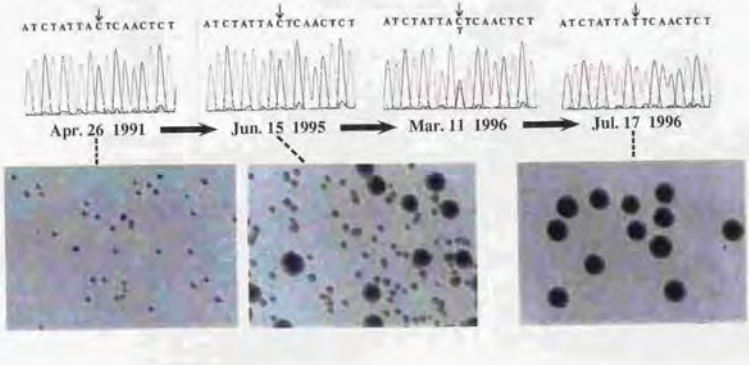



Fig. III-2 *"Microevolution" of WL cells in "wild-type" culture in our laboratory as demonstrated by direct sequencing and colony size.*

"Wild-type" cells, which had been maintained under photoautotrophic conditions, were stored at -80 C in 1991, 1995 and 1996. These frozen cells were thawed, propagated onee in a batch culture and then analyzed. Upper panels show direct sequencing of position 193 of *pmgA* and its vicinity. Lower panels show colonies grown at 370 $\mu Em^{-2}s^{-1}$ for 3 days.

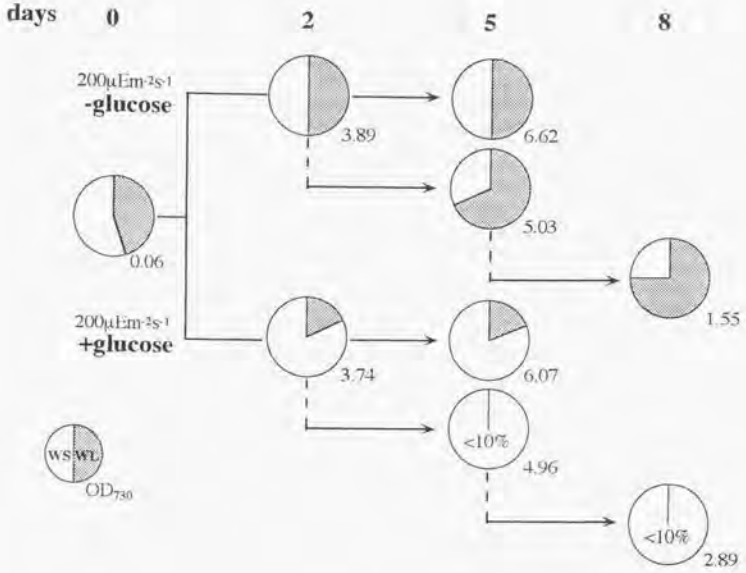



Fig. III-3 *Effects of growth conditions for mixed liquid culture on the ratio of the WL to WS genotype as determined by direct sequencing.*

The ratio was expressed as a pie chart, while cell density was shown as $OD_{730}$ under each pie chart. A horizontal line indicates each batch culture, while a dotted line indicates the inoculation of the following batch culture.

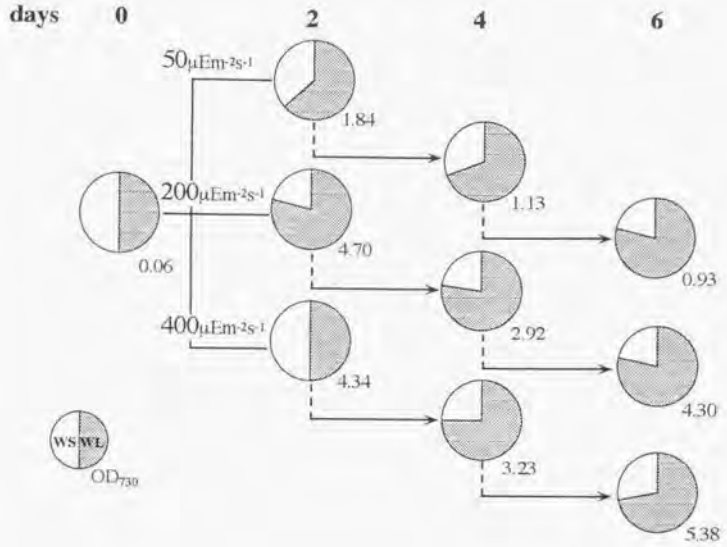



Fig. III-4 *Effects of light intensities for mixed liquid culture on the ratio of the WL to WS genotype as determined by direct sequencing.*

The ratio was expressed as a pie chart, while cell density was shown as $OD_{730}$ under each pie chart. A horizontal line indicates each batch culture, while a dotted line indicates the inoculation of the following batch culture. Cultures were transferred to a new medium every two days.

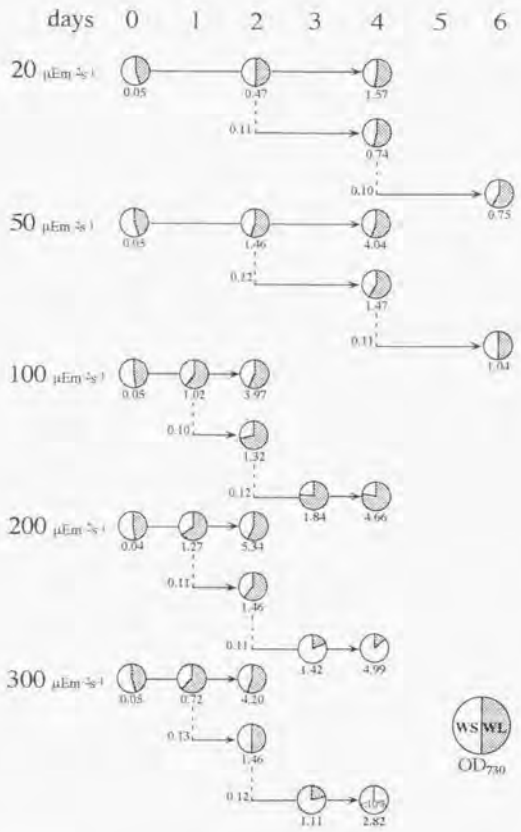



Fig. III-5 *Effects of light intensities for mixed liquid culture on the ratio of the WL to WS genotype as determined by direct sequencing.*

The ratio was expressed as a pie chart, while cell density was shown as $OD_{730}$ under each pie chart. A horizontal line indicates each batch culture, while a dotted line indicates the inoculation of the following batch culture. Cultures under high light were transferred to a new medium every 24 h.

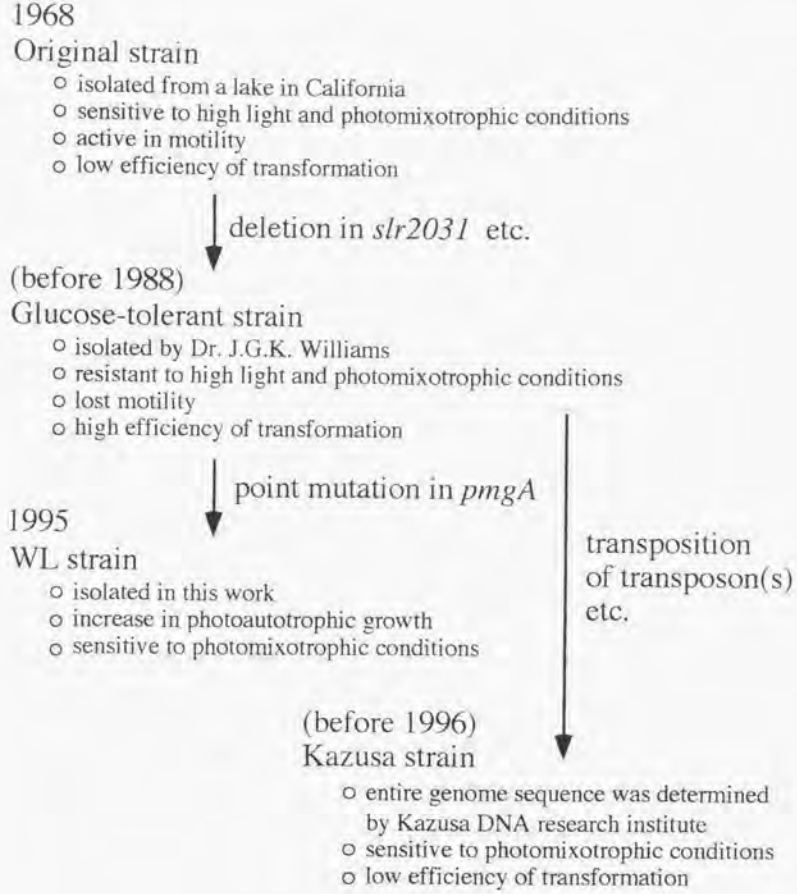



Fig. III-6 *"Microevolution" of Synechocystis sp. PCC 6803 in laboratory conditions.*

## General Discussion

As described in General Introduction, many years of assiduous effort of physiologists have brought us quite a lot of knowledge of acclimative responses of photosynthetic organisms to light environment. They optimize a whole set of the photosynthetic apparatus by regulation of the various processes such as the turnover of the existing structural-functional organization and the biosynthesis-assembly of new components. However, the mechanism by which the organisms recognize a change of light environment and eventually translate it into biosynthetic activity and assembly of new components is still unknown.

In this study, I got a clue to the molecular mechanism for high-light acclimation. In Chapter 1, I have reported the identification of a novel polymorphic gene, *pmgA*, which affected the colony size of wild-type *Synechocystis* sp. PCC 6803 under photoautotrophic conditions. I have also demonstrated that *pmgA* was essential for photomixotrophic growth but its mutants showed enhanced growth under photoautotrophic conditions. In Chapter 2, I have examined the phenotype of *pmgA* mutants in detail, and showed that *pmgA* is specifically involved in the regulation of photosystem stoichiometry, one of the responses typically observed upon the shift to high light. The lack of photosystem modulation in the mutants resulted in cells with elevated PSI levels and enhanced rates of photosynthesis. This seems to be the reason for the enhanced growth of *pmgA* mutants under photoautotrophic conditions. I have also shown that expression of *pmgA* transcripts was activated upon the shift to high light. In Chapter 3, I have examined the significance of *pmgA*-mediated acclimation process(es) using

direct sequencing method. I have shown that the *pmgA* mutant with a base replacement spontaneously appeared in wild-type culture a few years ago and completely drove the wild-type cells away from the culture. However, it was revealed that the mutant could not survive under the prolonged stress conditions of high light as well as under photomixotrophic conditions. I would like to point out that though *pmgA* was not required for the photoautotrophic growth in rather constant laboratory conditions, it must be indispensable for survival in natural environment which always fluctuates. At present, two terms "acclimation" and "adaptation" are strictly distinguished. "Acclimation" means fitting to the environment without genetic change, whereas "adaptation" means that with genetic change. I found that *pmgA* is involved in the acclimation process by changing photosystem stoichiometry. Furthermore, I found that *pmgA* itself had been changed in wild-type *Synechocystis* to adapt to the laboratory conditions. In this sense, *pmgA* is involved in not only acclimation but also adaptation. It must be a rare case and worthy of special mention.

Physiological significance of the high light response that adjusts photosystem stoichiometry has not been fully established although the adjustment is wide spread in photosynthetic organisms. For example, it was simply stated in a review (Anderson et al., 1995) that adaptation of the photosystem stoichiometry serves to regulate the distribution of excitation energy between the photosystems and correct any imbalances. This contrasts to many other responses of high light acclimation such as the reduction in pigments and antenna size and the increase in $CO_2$ fixation activity, which can be easily recognized as avoiding photoinhibition (Björkman, 1981; Anderson et al., 1988). Here, I proposed the same

physiological role for the adjustment of photosystem stoichiometry under high light based on my findings in this study. Figure GD-1 shows schematic representation of possible organization of the photosynthetic system and photosynthetic electron flows in *Synechocystis* sp. PCC 6803 at low or high light intensities. At low light intensities, where light capture is limiting, antenna size of photosystems is separately up-regulated by the size of phycobilisome for PSII and by the number of reaction center for PSI. Since the total light-harvesting capacity of PSII which has phycobilisome is larger than that of PSI in this case, the optimal ratio of PSII/PSI is about 0.5 (Fig. GD-1, upper panel). On the other hand, at high light intensities, where electron transport rather than light capture becomes limiting, absorption of excess light energy can be avoided by reduction of both antenna size and photosystem content. Furthermore, the ratio of PSII/PSI is also changed to about 1.0 in wild type (Fig. GD-1, middle panel). Conversely, the capacity of $CO_2$ fixation increases in order to keep the balance between energy supply and consumption. In spite of the up-regulation of $CO_2$ fixation and the down-regulation of antenna and photosystems, energy provided by high light illumination may be still too much to be consumed. This leads to photoinhibition of PSII via accumulation of electrons at its reducing side (Aro et al., 1993). Since electrons are supplied by water oxidation of PSII, the photoinhibition of PSII consequently balance the energy supply and consumption by down-regulation of the whole electron flow. In addition, the damaged PSII will be repaired by rapid turnover of reaction center D1 protein. Thus, photoinhibition of PSII is thought to be a part of protection mechanism against high light stress (Anderson et al., 1997). In the case of *pmgA* mutants, selective reduction of PSI content at high light is abolished

by defect of *pmgA*. Resulting higher abundance of PSI in *pmgA* mutants extracts more electrons from the PQ pool. As a result, photoinhibition of PSII was mitigated and the whole electron flow is enhanced even with unchanged content of PSII (Tables II-1 and II-3). This enhanced electron flow possibly stimulates $CO_2$ fixation rate for a short term. However, higher electron flow together with more PSI makes the acceptor side of PSI more reductive, leading to excess accumulation of electrons in ferredoxin/NADP pool (Fig. GD-1, lower panel). It is widely accepted that electrons generated from PSI react with oxygen to produce the superoxide anions, which are mostly scavenged by superoxide dismutase and ascorbate peroxidase (Asada, 1992; Herbert et al., 1992). However, the excess accumulation of electrons in the mutants stimulates the generation of reactive species of oxygen over the capacity of the scavenging system. This may give the oxidative damage to cellular components and finally lead to the loss of viability of the mutants under the prolonged stress of high light (Fig. III-5). Similar damage has already been demonstrated in higher plants as an irreversible photoinhibition of PSI (Sonoike and Terashima, 1994; Sonoike, 1996). Namely, PSI is selectively damaged under certain conditions where electrons are accumulated at the acceptor side of PSI over the capacity of scavenging system for reactive species of oxygen. Chilling sensitive plants such as cucumber receive this photodamage at low temperature due to a temperature-dependent loss of protection against the reactive species of oxygen. In conclusion, the adjustment of photosystem stoichiometry under high light conditions mediated by *pmgA* in *Synechocystis* can be reasoned as a physiological response to the high light stress: the decrease of the PSI content makes cells resistant against high light stress by reducing production

of the reactive species of oxygen, which are otherwise lethal under prolonged high light stress. It would be very interesting to attempt to detect preferential accumulation of the reactive oxygen species under high light conditions in *pmgA* mutants.

Then, why are *pmgA* mutants unable to grow under photomixotrophic conditions? Can this phenomenon be explained by the lack of the regulation of photosystem stoichiometry in mutants? To answer this question, I performed a preliminary measurement of the low temperature fluorescence spectra of wild-type and *pmgA* mutant cells with or without glucose. It was observed that wild-type cells grown under the photomixotrophic conditions showed much reduced chlorophyll content on a per cell basis (data not shown) and high ratio of PSII to PSI under 50 $\mu Em^{-2}s^{-1}$ (Fig. GD-2). On the other hand, *pmgA* mutants did not show any change in the photosystem stoichiometry under the same photomixotrophic conditions. Since these changes were almost the same as those under high light, addition of glucose are supposed to intensify the light stress even at 50 $\mu Em^{-2}s^{-1}$. This interpretation may be reasonable, if glucose provides cells with NADPH via oxidative pentose phosphate cycle (Pelroy et al. 1972), which eventually makes the acceptor side of PSI more reductive like the high light treatment (Fig. GD-3). Thus, the phenotype of *pmgA* mutants unable to grow under the photomixotrophic conditions could be also explained by the inability to reduce the PSI content.

There are no information about the location of *pmgA* product in the signal transduction pathway which leads to the adjustment of photosystem stoichiometry. Since *pmgA* does not have domains similar to known sensors or transcription factors, its product might work as signal transducer located

on the middle of signal transduction pathway. Then, from where *pmgA* receives a signal? As described in General Introduction section, electron carriers located between the acceptor side of PSII and the donor side of the cytochrome *b6/f* complex are considered to be a most likely candidate for a sensor for change of light environment. Since the rate of photon absorption by PSII exceeds the rate of plastoquinol oxidation at the $Q_O$ site of the cytochrome *b6/f* complex upon the shift to high light, overreduction of such electron carriers near PSII can be a signal for the elevation of light intensities. However, this sensing mechanism which has been proposed by many groups does not seem reasonable in the case of regulation of photosystem stoichiometry mediated by *pmgA*. If the overeduction near PSII leads to the down-regulation of PSI content as acclimation to high light, the electron carriers near PSII are supposed to more reduced by such a regulation. Since *pmgA* modulates the photosystem stoichiometry by specific regulation of PSI, the electron carriers near PSI may also work as a sensor for high light intensities in order to adjust the photosystem stoichiometry in cyanobacteria. Though this possibility has never been discussed before, it may be meaningful to investigate whether or not the electron carriers around PSI such as a ferredoxin/thioredoxin system are involved in the signal transduction for acclimative responses.

Since *pmgA* is the first gene identified as a specific regulatory factor for acclimation processes, I believe this study will become a breakthrough for molecular dissection of the acclimative processes. At this stage, there are no established information about sensors or transducers in acclimation to environmental changes as described in General Introduction section. However, by examination of the conditions under which expression of

*pmgA* is inducible, we might know the sensing mechanism for modulation of photosystem stoichiometry at high light. Furthermore, by isolating factors which interact with *pmgA* within the signal transduction network, we might get a clue to the process of signal transduction. It is known that the regulation of photosystem stoichiometry also occurs under conditions such as different light qualities or $CO_2$ availability (Fujita et al., 1985; Murakami et al., 1997). By investigation of whether or not *pmgA* mutants show the regulation of photosystem stoichiometry under such conditions, we may dissect the regulation network of these acclimation processes. Moreover, survey of a *pmgA* homolog in other cyanobacteria and/or plants might reveal a universal mechanism for light acclimation in global photosynthetic organisms.

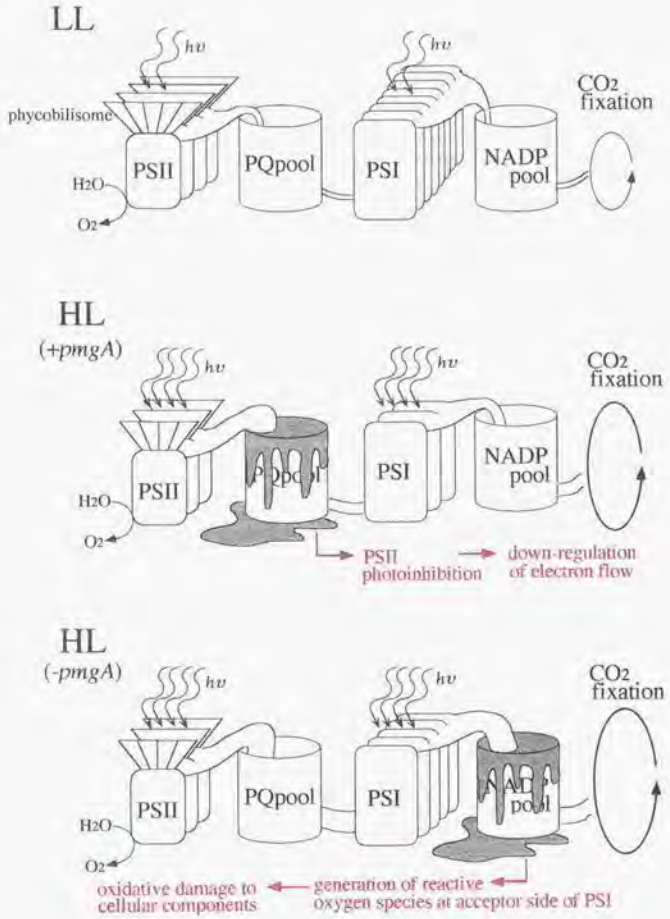



Fig. GD-1 *Schematic representation of the photosynthetic apparatus in Synechocystis sp. PCC 6803 with or without pmgA at different light intensities.*

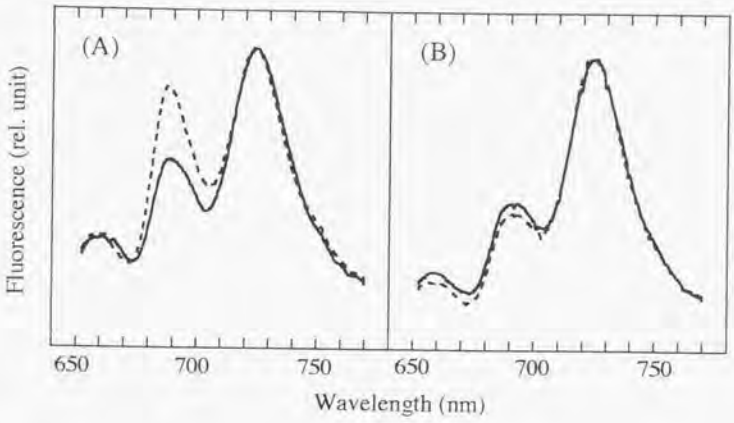



Fig. GD-2 *Low temperature (77K) fluorescence emission spectra of cells.*

(A) Fluorescence emission spectra at 77K of WS.
(B) Fluorescence emission spectra at 77K of WL.
Spectra of cells grown at 50 $\mu Em^{-2}s^{-1}$ without glucose (solid line) and cells grown at 50 $\mu Em^{-2}s^{-1}$ with glucose (dashed line) were normalized at the 725 nm peak of PSI.

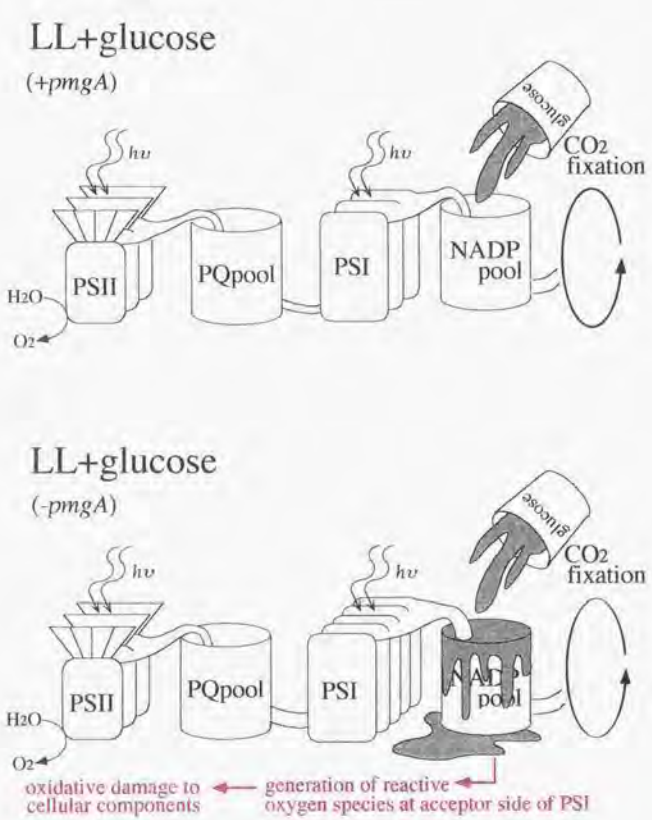



Fig. GD-3 *Schematic representation of the photosynthetic apparatus in Synechocystis sp. PCC 6803 with or without pmgA under photomixotrophic conditions.*

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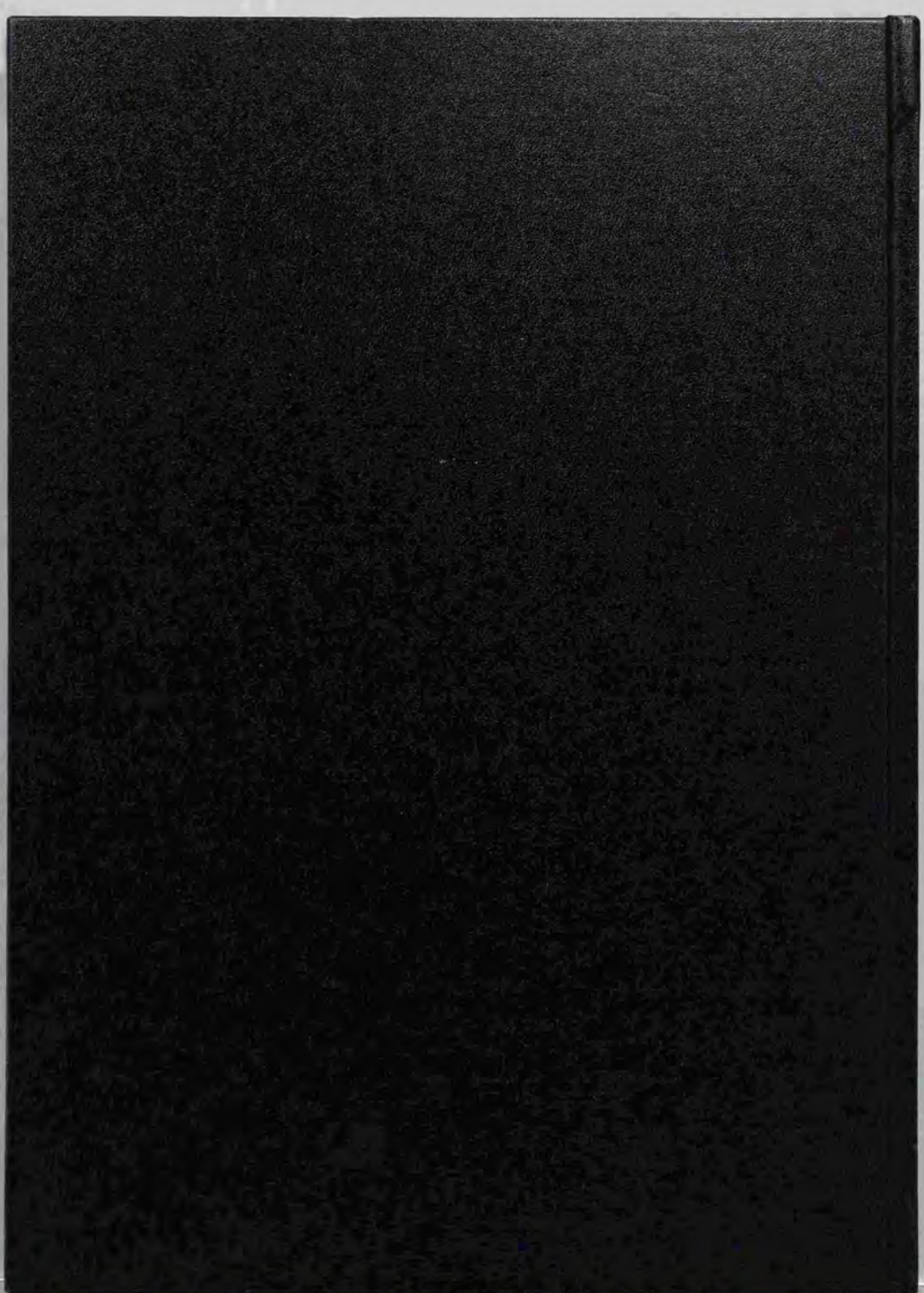

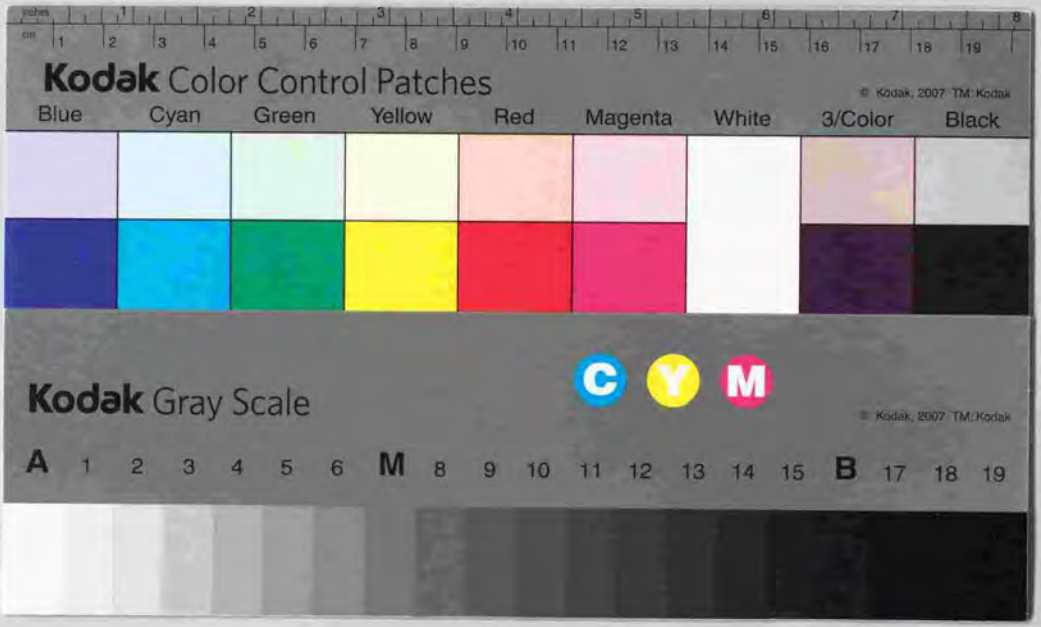
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