High-light response of the cyanobacterium *Synechocystis* sp. PCC 6803

～Role of *sll1961* gene during acclimatory process～

シアノバクテリア*Synechocystis* sp. PCC 6803の強光応答

—順化過程における*sll1961*遺伝子の役割—

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シアノバクテリア *Synechocystis* sp. PCC6803 の強光応答
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[序論]

光合成生物にとって過剰な光エネルギーの供給 (強光)は、光合成電子伝達鎖の過還元状態を引き起こし、光合成装置における活性酸素種の生成を誘発する為、非常に危険である。そこで、シアノバクテリア *Synechocystis* sp. PCC 6803 は、光環境の変動に対し、光吸収量の抑制、光合成電子伝達の調節、CO_2 固定系の調節、活性酸素消去系の誘導等、様々な応答機構を働かせる。このような機構の一つとして、光化学系量比調節機構が知られている。この機構により、強光下で光化学系 I が選択的に抑制され、光化学系 II に対する光化学系 I の量（系 I／系 II 比）が低下する。光化学系量比調節機構に関与する因子ついては、推定転写因子 *sll1961* などが知られているが、*sll1961* が光化学系量比調節機構にどう関与するのかは、全く明らかでない。さらには、生育光環境は、光合成系以外にも大きな影響を与えることが予想されるが、これについても実際に得られた知見はほとんどないと言ってよい。そこで、本研究では、シアノバクテリア *Synechocystis* sp. PCC 6803 を材料とし、１．細胞の形態に対する強光の影響と光化学系量比調節の関係、２．　*sll1961* の光化学系量比調節機構における位置、の二点について解析した結果を報告する。

[結果・考察]

１．細胞の形態に対する強光の影響と光化学系量比調節の関係

強光に際して細胞内の状態は大きく変化する。多くの生物種では、そのような生理状態の変化に伴い細胞形態が変化する例がある。本章では、光化学系量比調節と細胞形態の関わりを検証することとした。

(1) 強光下での細胞形態の変化

弱光 (20 μmol $m^{-2}s^{-1}$)下で生育した野生株を強光 (200 μmol $m^{-2}s^{-1}$)に移行し、経時的に位相差顕微鏡で観察し、細胞直径を測定した。その結果、野生株の細胞サイズは、強光移行後大きな変化を示すことがわかった（図　1）。まず、強光移行後 6 時間までは、細胞直径は殆ど変化を示さず、その後 12 時間までに細胞直径は著しく増加し、最大で移行前の 25%程度大きくなった。その後、48 時間まで細胞直径は緩やかに減少し、48 時間以降はほぼ一定の値を維持した。この強光定常レベルでの細胞直径と弱光培養株の細胞直径は有意に異なっていた。また、強光移行後 12 時間における最大直径も、強光順化後の定常レベルの直径と有意に異なっていた。一方、強光に順化した細胞を弱光に移すと、弱光順化していたもとの大きさまで細胞直径が減少し、この際には一過的な細胞直径の変動は見られなかった。以上の結果から、シアノバクテリアにおいては、細胞のサイズを光環境に合わせて変化させることが示唆された。細胞のサイズを外界の光強度に依存して変化させる意義については、現在のところ明解な答えはない。しかし、細胞のサイズが大きくなると、自らの細胞内で光合成色素同士が被陰効果を示し、効率的な光合成を阻害すると考えられる。その為、光合成が生育の律速段階となる弱光下では、大きな細胞は不利になると考えている。

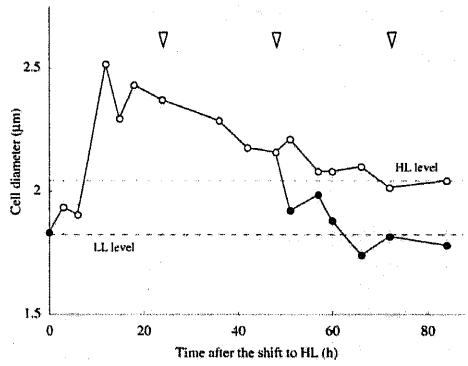

図 1. 強光移行後の細胞直径の変化　弱光生育細胞を黒丸で、強光生育細胞を白丸で表した。

(2) 光化学系量比調節欠損株の形態変化

強光移行後 12 時間前後に見られる細胞の一過的な巨大化は、何に起因するのだろうか。強光下から弱光下への移行時には一過的な変化は見られなかったことから、強光ストレスが細胞の大きさの変

化を引き起こしている可能性が考えられる。そこで、強光下でよりストレスを受けやすくなっていると考えられる強光応答関連遺伝子の欠損株の細胞形態を観察することとした。光化学系量比調節に欠損をもつ遺伝子破壊株、*pmgA* 破壊株, *sll1961* 破壊株, *nblA* 破壊株, *ccmK2* 破壊株, *slr2057* 破壊株, *slr1916* 破壊株, *ctaEI* 破壊株, *ctaCI* 破壊株, *slr0645* 破壊株を強光移行後 12 時間、24 時間において観察し、細胞直径を比較した。強光移行 12 時間後、これらの遺伝子破壊株のうち *pmgA* 破壊株, *sll1961* 破壊株, *nblA* 破壊株, *ccmK2* 破壊株, *slr2057* 破壊株, *slr1916* 破壊株の 6 株 (group A) が野生株よりも有意に大きい細胞直径を示した（図２）。また、これらの破壊株は、強光移行前の細胞直径に対する強光移行 12 時間後の細胞直径の増加率が野生株より高いこともわかった。これに対し、前述の group A 以外の 3 つの遺伝子破壊株 (group B) は、強光移行 12 時間後においても野生株と同レベルの細胞直径を有していた。では、これら 2 つのグループにおける細胞直径の変化の違いは、何を意味するのだろうか？強光移行 24 時間後において、これら９株のクロロフィル含有量を測定した。その結果、以前に報告がある通り、group A に属する６株はいずれも、野生株より高いクロロフィル量を示し、これに対し group B に属する３株は野生株と同じか、より低いクロロフィル量を示した。シアノバクテリアにおいて、クロロフィル分子の約 90% が系 I に配位している為、group A に属する遺伝子破壊株は系 I を多く有すると言える。以上の結果を併せて考えると、強光下において系 I を多く有することが、細胞の巨大化を引き起こした可能性が高い。過剰な系 I による細胞の巨大化を説明する理由として、酸化ストレスの影響が考えられる。光合成生物において、活性酸素種の主な発生部位が系 I の還元側にある為、group A の破壊株は過剰な酸化ストレス下におかれていると考えられるからである。酸化ストレスがなぜ細胞の巨大化を誘導するのかについては明らかでないが、窒素欠乏時には窒素欠乏応答欠損株（*nbl* 破壊株）の細胞直径が大きくなることが報告されており、*Synechocystis* sp. PCC 6803 のストレス下における細胞サイズの増加は様々なストレス下で共通に見られる事象である可能性がある。またこれまでの結果から、仮に細胞サイズをストレスの指標としてとらえた場合、強光下のシアノバクテリアにとっては、光化学系量比のバランスを維持することよりも増して、系 I の量を適切に管理することが重要であることを意味していると考えられるだろう。

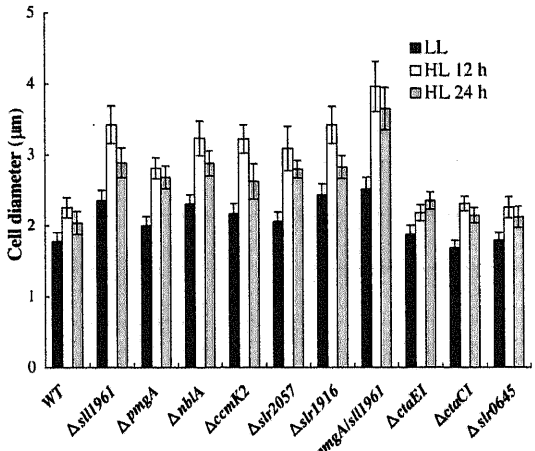

図 2. 強光下における光化学系量比調節欠損株の細胞直径の変化

２．*sll1961* の光化学系量比調節機構における位置

Sll1961 は Herix-turn-herix ドメインをもつことから、Sll1961 を介した光化学系量比調節が、転写調節を介して行われる可能性が高いと考えた。先行研究で行われた *sll1961* 破壊株での網羅的発現解析の結果から、4 つの遺伝子を Sll1961 による転写調節の標的候補として取り上げた。標的候補遺伝子破壊株は、強光下での光化学系量比調節に部分的な欠損を示した。そこで、標的候補遺伝子の上流域の DNA 断片と Sll1961 の組み換えタンパク質を用いてゲルシフトアッセイ (EMSA)を行った。その結果、全種の DNA 断片に対し、高タンパク質濃度条件下で妥当なサイズの DNA-タンパク質複合体形成が観察されたが、配列特異性を確認することはできなかった。また、強光生育のシアノバクテリア由来の粗タンパク質液を用いた EMSA では、*slr0364* と *slr2057* に対する DNA 断片において、組換えタンパク質を用いた EMSA で見られた複合体とほぼ同じサイズのシフトが見られた。Sll1961 の転写標的遺伝子を特定するには至っていないが、いくつかの条件において、適当なサイズの DNA-タンパク質複合体形成が見られたことから、Sll1961 が反応条件によって、これらの DNA 断片に対して特異性を示す可能性は残されていると考えている。Sll1961 が分類される GntR 型転写調節因子は、様々な補因子によって活性調節を受けることが知られており、Sll1961 の DNA 結合能が低いことは、実験条件における補因子の欠如によるものである可能性が高い。今後の Sll1961 を介した光化学系量比調節機構の解明のためには、補因子の同定が必要なのではないかと考えている。

Contents

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Abbreviations

HL	High light
LL	Low light
PSII	photosystem II
PSI	photosystem I
PCC	Pasteur culture collection
F	Fluorescence
OD	Optical density
EMSA	Electrophoretic mobility shift assay

Summary

Regulation of photosystem stoichiometry is the important acclimatory process against high-light stress. Although the *sll1961* mutant is known to be almost-entirely defective in the regulation of photosystem stoichiometry under high-light, the direct involvement of the Sll1961 in this PSI/PSII regulatory process is unknown. In this study, I tried to shed some light on the questions that (i) how high-light as well as lacks of regulation activity of photosystem stoichiometry affect cyanobacterial cell morphology, and (ii) how the photosystem stoichiometry is regulated through the transcriptional regulation of Sll1961.

First, I analyze the change in cell size of the cyanobacterial cells under prolonged high-light illumination with phase contrast microscopy. Cell size of the wild type showed transient increase after the shift to high-light, and showed a shift to fixed size under prolonged high-light illumination. It is shown that cyanobacteria modulate its cell size under high-light. The transient increases of cell size were significantly high in the mutants that have increased level of PSI. It may suggest that the high PSI amount under high-light cause severe stress, and the stress condition leads the cell size enlargement in *Synechocystis*.

Second, I examined the transcriptional regulation by Sll1961, which has a herix-turn-herix domain on its N-terminus. The genetic disruption of the putative targets of Sll1961, i.e. the *slr0364*, *slr0366*, and *slr2057* genes, slightly increased PSI/PSII ratio under high-light. From the EMSA (electro mobility shift assay) with His-tagged Sll1961 and the promoter segment of putative targets, I could detect interactions in reasonable molecular size, but the sequence specificity was not observed. Further investigation can be clear which genes Sll1961 regulates.

General Introduction

Light is essential to photosynthetic organism because of their energy production or carbon fixation. Light environment change drastically in nature, so photosynthetic organism must acclimate to ever changing light environment. In response to changing light intensity, they modulate capacities of light harvesting, photosynthetic electron transport, CO_2 fixation Melis et al., 1991; Anderson et al., 1995; Walters et al., 2005), and scavenging system of reactive oxygen species (Grace and Logan 1996; Niyogi 1999). If energy supply exceeds its capacity for dissipation, particularly under high-light conditions, the photosynthetic electron transport components could become relatively reduced. This may result in excess production of reactive oxygen species (ROS) leading to severe damage to various kinds of cellular processes (Asada 1994). Thus, absorption of excess light energy must be avoided under high-light condition.

In high-light acclimatory process, cyanobacteria reduce the amount of both antenna complex and photosystem content. The decrease in photosystem content is typically observed under high-light condition and the main target of down-regulation is not PSII but PSI (Murakami and Fujita 1991; Hihara et al. 1998). This reduction of PSI content that alters the ratio of PSI/PSII is called the regulation of photosystem stoichiometry. The regulation of photosystem stoichiometry is known to be essential for the survival of cyanobacteria under prolonged high-light condition (Hihara et al. 1998, Fujimori et al. 2005).

It has been well documented that the regulation of photosystem stoichiometry is the important acclimatory process to high-light. However, (i) the importance of photosystem stoichiometry regulation for cellular processes other than photosynthesis , and (ii) the detailed mechanisms of regulation of photosystem stoichiometry, are both practically unknown. In this

study, I reported about (i) the effect of high-light on the cell morphology of the wild type and the mutants that are defective in the regulation of photosystem stoichiometry, and (ii) the transcriptional regulation by Sll1961, which is the key factor of photosystem stoichiometry.

Chapter I

~The cell size control during high-light acclimation,
and the importance of PSI/PSII regulation ~

Introduction

Too strong light causes severe stress to photosynthetic organisms. Under high-light condition, cyanobacteria exert various sorts of stress response mechanisms, modulation of capacities of light harvesting, photosynthetic electron transport, CO_2 fixation (Anderson 1986; Melis 1991; Anderson 1995; Walters 2005), and scavenging system of reactive oxygen species (Grace and Logan 1996; Niyogi 1999).

As mentioned above, cyanobacteria change its physiological state under high-light. It is known that cell morphology reflects physiological state of the cell in many unicellular organisms. Although the dynamic changes of cell physiology under high-light condition has been extensively studied, little is known for the cell morphology under high-light stress in cyanobacteria.

The regulation of photosystem stoichiometry is brought about in order to adjust and optimize the excitation of the two photosystems under different qualities of light (Fujita 1997), or to protect the cells from photodamage under high photon flux densities (Hihara et al. 1998, Sonoike et al. 2001). However, it has not been studied about the importance of the regulation of photosystem stoichiometry for cellular activity other than photosynthesis. In this chapter, I studied the effect of high-light stress on cell morphology of the cyanobacterium, *Synechocystis* sp. PCC 6803, as well as the effect of irregular PSI/PSII ratio on the changes of cell morphology under high light condition.

Results

Change in cell-size in the wild type after the shift to high-light

To investigate how cell morphology of the wild type strain of the cyanobacterium, *Synechocystis* sp. PCC 6803, changes under high-light, I compared the low-light-grown cells with high-light-grown cells by phase-contrast microscopy (Figure 1). I observed a small change in the cell-size of the wild type cells of *Synechocystis*. The cells grown under high-light (200 μmol $m^{-2}s^{-1}$) looked slightly larger compared with the cells grown under low-light (20 μmol $m^{-2}s^{-1}$). It may suggest that high-light affects not only photosystem but also the cell morphology of *Synechocystis*. In order to understand the change of cell-size in the wild type under high-light, I observed the time-course of cell-size after the shift from low-light to high-light in the wild type (Figure 2). The cell-size was measured as cell diameter under the phase-contrast microscopy, the average of 20 cells at each time point was calculated. Since the increase of the cell density tends to decrease the photon flux density the each cell receive, the cell culture was diluted every 24 hours to keep the high light condition (arrowheads in Figure 2). Upon the shift to high-light, the cell size was unchanged for 6 hours, and then cell size significantly increased up to about 125% of that of low-light-grown cells in 12-24 hours. With continuous high-light illumination, cell size gradually decreased to 115% of that of low-light acclimated cells in 48 hours. Up to 60 h, cell-size remained, under high-light condition to be, at the stationary level at 115 % of that of low-light-grown cells. Thus, cell size of *Synechocystis* varies depending on the growth light intensity. The acclimatory process takes at least 6 hours, and high light grown cells are larger than low light grown cells by 15%. In addition, there seems to be a transient over-increase of cell size at 12-24 hours after the shift to high-light.

Is the cell size controlled in response to light intensity? Next, I examined the change of cell size when the cells were shifted from high-light to low-light (Figure 2, closed circle line). The wild type cells grown under high-light for 48 hours were shifted to low-light, and cell size was determined with phase-contrast microscopy. Upon the shift from high-light to low-light, cell-size of the wild type decreased for about 24 hours, and came into the same level before the shift to high-light. It should be also noted that the transient over-decrease (or increase) of the cell-size, which was observed in the shift from low-light to high-light, was not observed. The difference of the cell-size between the cells grown for 84 hours under high-light and cells grown under low-light or cells at the transient phase (i.e. cells at 12 hours after the shift to high light) was statistically significant. This result suggests that the optimal cell-sizes vary according to the light intensity, and the cells of the wild type *Synechocystis* control their cells into the optimal size during the acclimation to high-light. Since the transient over-increase of cell size was detected only after the shift to low-light to high-light, It is indicated that the high-light exposure has another effect than the simple acclimation of cell-size to photon flux densities.

Cell size control of the mutants, which are defective in the regulation of PSI/PSII

As mentioned above, it was shown that the wild type cells changes their size into an optimal level in acclimation to high-light. However, it is still unknown the reason why the wild type cells enlarged so drastically and suddenly after the shift to high-light. Because the temporal effect of changing light intensity on the cell size was detected only after the shift to low-light to high-light, not after the shift to high-light to low-light, the transient increase of the cell size looks like a result of a stress caused by the shift to high-light. To verify this

hypothesis, I compared cell size of the wild type with that of the *sll1961* mutant, which is known to be defective in one of the high-light acclimatory response, regulation of photosystem stoichiometry, with microscopy (Figure 3). The *sll1961* mutant is thought to be in stressful condition, because of its defective acclimation to high-light. Even under low light condition, cells of the *sll1961* mutant are significantly larger than the wild type (Figures 1 and 3). After 12 hours under high-light, the cell size of the *sll1961* mutant further increased. Percentage of the cell size enlargement upon the shift from low-light to high-light in the *sll1961* mutant looks like higher than that in the wild type (Figure 1 and 3).

In order to study the effect of high-light stress on the cell size in detail, I observed all the nine mutants, which are known to show irregular PSI/PSII ratio under high-light, and compared with the wild-type in cell diameter at 0, 12 or 24 hours after the shift to high-light (Figure 4). Cells of the *sll1961*, the *nblA*, the *ccmK2*, and the *slr1916* mutant strains were significantly larger under both low-light and high-light condition as compared with wild type cells. The *pmgA* mutant and the *slr2057* mutant showed significant increased in the cell-size under high-light, but the cell size under low-light condition is not so much different from that of the wild type cells. Percentage increase of the cell size upon the shift to high light in the wild type, *sll1961* mutant, the *pmgA* mutant, the *nblA* mutant, the *ccmK2* mutant, the *slr2057* mutant, and the *slr1916* mutant are 25%, 45%, 40%, 40%, 48%, 49%, and 40%, respectively at 12 hours after the shift to high-light. Since the cells of wild type became 25% larger in this condition, degree of cell size increase upon the shift from low-light to high-light was significantly higher in these six mutants. In addition, I also measured the cell diameter in the *pmgA/sll1961* double mutant, the *pmgA/sll1961* mutant showed remarkably large cell size than the wild type, even larger than the cells of the other group I mutants. In contrast, the

ctaEI mutant, the *ctaCI* mutant, and the *slr0645* mutant showed same cell size as the wild type under low-light condition, and the increase of the cell size upon the shift to high-light condition was also comparable with the wild type cells. Thus, there are two groups in these mutants, which show different cell size depending on the light condition.

Here, I will tentatively call the six mutants that showed larger cell size than the wild type under high-light as group A, and the three mutants that showed similar cell size to the wild type under high-light as group B. Although the mutants in group B were reported to have defect in PSI/PSII regulation under high-light, these mutant showed normal cell size control under high-light. To confirm the status of PSI/PSII under high light in group B mutants, I measured chlorophyll fluorescent spectra at liquid nitrogen temparture (77K). It is well known that the ratio of fluorescent peak height at 725 nm to the peak height at 695 nm is a good measure for PSI/PSII ratio (Figure 5A). As reported earlier, both group A and group B mutants showed significantly higher PSI/PSII ratio as compared with the wild type (Figure 5B). This supports that the difference in the cell sizes under high-light between group A and group B is not due to the insufficient acclimation of the photosystem stoichiometry, but due to the qualitative difference between the two groups.

It is reported that the mutants with abnormal PSI/PSII regulation under high-light condition could be classified into two groups, based on the chlorophyll content and viability under photomixotrophic condition (Ozaki et al., 2007). It is known that chlorophyll content in the cell of cyanobacterium is a good index of PSI abundance, thus giving information of the cause of the change in photosystem stoichiometry. I measured the absorption spectra of whole cells of the wild type and the mutants suspended in BG-11 media, then calculated the chlorophyll content per OD_{750} (Figure 6). As a result, all of the mutants contained in group A

showed significantly high chlorophyll content than the wild type under high-light. This result clearly indicated that the increase of PSI/PSII ratio is the result of increased PSI content as reported earlier (Ozaki et al., 2007). On the other hand, the mutants in group B showed low or regular chlorophyll contents under high-light. This result is also consistent to Ozaki et al. (2007), indicating that the change in PSI/PSII ratio should be cased by the result of decreased PSII content. Thus, I conclude that the mutants with higher PSI content under high-light tend to become larger in cell size, while the mutants with lower PSII content under high-light condition respond to high-light with similar manner with wild type cells.

Furthermore, the *pmgA/sll1961* mutant showed high PSI/PSII ratio (Figure 5B), and high chlorophyll content under highlight (Figure 6). Although the *pmgA/sll1961* mutant showed significantly higher cell size than the other group I mutants, the PSI content of the *pmgA/sll1961* mutant was similar to the other group I mutants.

Discussion

Change of cell-size in the wild type after the shift to high-light

Some cyanobacteria, especially filamentous ones, are known to show some morphological change upon environmental changes or gene disruption. However, as a typical unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 has been treated as an organism that shows quite constant cell morphology. Usually, we determine the cell number of the culture by measuring OD_{730} (i.e. cell scattering). The coefficient of the conversion of OD_{730} to cell number should depend on the optical setting of spectrometer, but the same value would be used for the same spectrometer irrespective of the strains of *Synechocystis*. However, my study presented here demonstrate that the cell morphology, i.e. cell scattering, was largely affected by gene disruptions as well as by the growth light condition, leading to a vexing question on the estimation of the cell number from measurement of optical density.

For example, we often use a parameter, chlorophyll content per cell, to express the antenna function of the cyanobacterial cells. To determine this parameter, we measured OD_{730} of the cell culture, as well as the chlorophyll content. The measurement of the OD_{730} is based on the fact that cell scattering that could be estimated as the decrease of optical density is proportional to the number of cells. However, there is a prerequisite for that estimation: the size of the cell should be the same for the proportional relationship. Actually, the optical density also depends on the cell shape and cell size. The relationship between the optical density and the size of particle is rather complicated. From the physical aspect, scattering can be classified into three types: Rayleigh scattering, Mie scattering and diffraction scattering. Rayleigh scattering is applied when the size parameter, which is the ratio of particle size and light wavelength, is less than 0.1, while other two are applied when size parameter is about

unity and more than 10, respectively. Since the cell size of *Synechocystis* sp. PCC 6803 (about 1 μm) is comparable with the wavelength of visible light (0.4-0.7 μm), Mie scattering theory should be applied for the cell scattering of the cyanobacteria. The precise theory of Mie scattering is very complex but we can assume that scattering is proportional to the surface area of the particle (i.e. proportional to the square of the diameter) as a rough approximation. Chlorophyll contents of the cells are assumed to be proportional to the area of thylakoid membranes. Since the thylakoid membranes takes concentric spherical shape within the cells of *Synechocystis* sp. PCC 6803, the area should be proportional to the square of the cell diameter if we assume the number of the thylakoid layer does not change. Under such assumption, the chlorophyll content per OD_{730} would not be affected by the simple enlargement of the cell size. Needless to say, the exact relationship depends on the actual cell morphology and more detailed structural analysis by e.g. electron microscopy would be necessary.

For first six hours from the shift to high light condition, the *Synechocystis* cell did not show any change in size (Figure 2). Interestingly, it took also about six hours for *Synechocystis* cells to acclimate to high-light, judging from the change in photosystem stoichiometry (Hihara et al. 1997, Fujimori et al. 2005). Thus, the change in cell size would be also an acclimatory response to high-light. It should be also noted that the doubling time of this organism under high-light condition is several hours, which agrees well to the above mentioned time to acclimate to high-light.

I assume that the transient increase of the cell size around 12 hours after the shift to high-light observed in Figure 2 is the result of high-light stress rather than simple acclimation to high photon flux densities, since the transient change of the cell size was not observe upon

the shift to low-light from high-light. The "meaning" of the stress in the cell size will be discussed in the next section.

Apparently, the steady state cell size is different between low-light acclimated cells and high-light acclimated cells. Adaptive significance of this phenomenon is not clear right now. However, one can speculate that the large cell size could become a physiological and ecological disadvantage for the cells, since the packed photosynthetic pigment within the cells would be more self-shaded with each other in larger cells. However, such disadvantage would not be much problem, when photon flux density in the environment is high. This may explain the different ecological niche in cell size for different light condition. This issue would be an interesting target of the next study.

Cell size control of the mutants, which are defective in the regulation of PSI/PSII

Next, I considered about the temporary enlargement of cell size under high-light illumination for 12 hours, which could be triggered by high-light stress. I compared cell size among the nine mutants that is defective in PSI/PSII regulation under high-light, since the failure of the photosystem regulation may lead to the enhanced stress under high-light. It is reported that eight mutants, the *pmgA* mutant, *sll1961* mutant, *nblA* mutant, *ccmK2* mutant, *slr1916* mutant, *slr2057* mutant, *ctaEI* mutant, *ctaCI* mutant and *slr0645* mutant have defects in the regulation of photosystem stoichiometry under high-light (Ozaki et al., 2007, and Sato's master thesis). These eight mutants are known to be classified into two groups. Group I contains the *pmgA* mutant, *sll1961* mutant, *nblA* mutant, *ccmK2* mutant, *slr1916* mutant. The mutants included in Group I show suppression of photomixotrophic growth and increased level of PSI under high-light. On the other hand, Group II mutants show normal level of PSI

under high-light and are insensitive to photomixotrophic growth condition. As for the *slr2057* mutant, I will show in chapter II that this mutant shows same phenotype with group I mutants.

In Figure 4, I observed that six mutants out of nine mutants showed significantly larger cell size, and the other three mutants showed indistinguishable cell size from the wild type at the time point of 12 hour from the shift to high-light. I noticed that all the six large mutants are the ones of group I, while the other thee mutants came under group II. Only the former six mutants, which showed large cell size at 12 hours under high-light, showed higher chlorophyll contents at 24 hours under high-light (Figure 6). This means that these mutants contain higher amount of PSI under high-light than the wild-type. This result is consentaneous with previous information (Ozaki et al., 2007). Since the main production site of reactive oxygen species is the acceptor side of PSI (Asada, 1994), high amount of PSI under high-light lead to oxidative stress condition in cells. I considered that that severe stress caused by the high PSI amount affect the cell morphology and dramatically increase the cell size of group I mutants. Effect of severe stress on cell morphology by the combination of some gene disruption and environmental stress were previously reported. In *Synechocystis* sp. PCC 6803, an *nbl* mutant, known to have defect in response to the nitrogen deficiency, shows 30% to 25% larger cell-size under nitrogen starved condition than the wild type (Li and Sherman, 2002). Perhaps the condition of the cells of the *nbl* mutant under nitrogen starvation may be different from the physiological status of the cell of photosystem stoichiometry mutants under high-light. However, it can be said that cells of the *nbl* mutant are in excessive stress conditions under nitrogen starvation just as in the case of the group I mutants under high-light. It is also consistent with the fact that the mutant of *ggpS* gene, wich is involved in the regulation of glycosyl glycerol, showed relatively larger cell-size under salt stress than the

wild type (Ferjani et al., 2003). In the absence of the *ggpS* gene, protection to salt stress would fail and the cells may experience severe stress condition like the one that induced in the Group I mutants under high-light.

To compare with the mutants of PSI/PSII regulation, I measured the cell size of the *sll1773* mutant, which shows regular PSI/PSII level under high-light (data not shown). The *sll1773* mutant showed normal cell size, and normal PSI/PSII ratio under high-light as well as under low-light. The *sll1773* gene encodes a pirin-like protein called PirA. Pirin is a recently identified protein in eukaryotes as a transcription cofactor or an apoptosis-related protein (Wendler et al., 1997). In *Synechocystis*, the induction of *pirA (sll1773)* is not related to cell-death, and the expression of *pirA* (*sll1773*) gene increased under high salinity, or other several stress conditions (Hihara et al., 2004). It is also reported that the expression of *pirA* is increased in the *sll1961* mutant only under high-light (Fujimori et al., 2005). If severe stress conditions induce the expression of *pirA*, it might be considered that the mRNA level of *pirA* increased under high-light in the other group I mutants, as in the *sll1961* mutant. That would be an interesting experiments that may lead to the understanding of stress condition induced in the mutants under high-light. This would be tested in near future.

I also measured cell diameter of the *pmgA/sll1961* double mutant (Figure 4). The *pmgA/sll1961* mutant showed remarkably large cell size than the wild type, even larger than the cells of the other group I mutants. Although the *pmgA/sll1961* double mutant showed an additive phenotype in the cell size, the PSI/PSII ratio and the chlorophyll content of this mutant was slightly lower than the s*ll1961* mutant or the *pmgA* mutant (Figure 5 & 6). It indicates that disruption of one of these two genes may be sufficient for the loss of PSI/PSII regulation, but the additional stress was induced by the double mutation. Thus, it may be

reasonable to assume that *pmgA* and/or *sll1961* functions in acclimatory process under high-light other than the regulation of photosystem stoichiometry.

In conclusion, I showed the cell size variation during acclamation to high-light in *Synechocystis*. It was suggested that the optimal cell-size differ according to the light intensity. Furthermore, it was indicated that the severe stress condition in the cells of cyanobacteria cause temporary substantial enlargement of cell size. And one of the causal factors may be the high amount of PSI under high-light. The cell-size has the potential to represent the severeness of the stress in the cyanobacterial cells. If the stress become a good indicator for stress, it could be indicate that regulation of the PSI amount is more important than the adjustment of excitation balance between PSI and PSII under high-light.

Chapter II

~The transcriptional regulation of Sll1961

on the PSI/PSII regulation under high-light~

Introduction

In high-light acclimatory process, cyanobacteria reduce the amount of both antenna complex and photosystem content. The decrease in photosystem content is typically observed under high-light condition and the main target of down-regulation is not PSII but PSI (Murakami and Fujita 1991; Hihara et al. 1998). This reduction of PSI that alters the ratio of PSI/PSII is called the regulation of photosystem stoichiometry. Through the recent studies, it was revealed that the transcriptional control of PSI genes was important for the decrease of PSI content during high-light acclimation. In *Synechocystis* sp. PCC 6803, coordinated down-regulation of genes encoding subunits of PSI (PSI genes) was observed upon the shift to high-light conditions preceding the decrease of PSI content (Hiharaet al. 2001, Huang et al. 2002, Muramatsu and Hihara 2003, Tu et al. 2004). It was also shown by a pulse-labeling experiment that the light-induced modulation in the translation rate of a reaction center subunit of PSI, PsaA protein, closely parallels the transcription rate of the *psaAB* genes (Herranen et al. 2005).

To the present, it was reported that several mutants have defects in the regulation of PSI/PSII ratio upon the shift to high-light. The physiological significance of changing ratio of PSI/PSII during high-light acclimation has been demonstrated by the characterization of the two mutants of *Synechocystis* sp. PCC 6803, i.e. disruptants of *pmgA* (*sll1968*) and *sll1961*, both of which have defect in decreasing their PSI content during high-light acclimation (Hihara et al. 1998, Fujimori et al. 2005). The decrease in PSI content during acclimation to high-light seems to be regulated through the repression of mRNA levels of PSI genes, judging from the data of whole genome DNA microarray (Hihara et al. 2001), Furthermore, the defect in repression of the transcriptional level of the *psaAB* genes (PSI genes) is likely to cause the

aberrant accumulation of PSI in *pmgA* disruptant under prolonged high-light condition (Muramatsu and Hihara 2003). In contrast to the *pmgA* mutant, it was shown that the transcriptional level of *psaA* drastically decreased upon the shift to high-light, and was continuously suppressed in the disruptant of *sll1961* as well as in the wild type. More recently, other six mutants that might be defective in the regulatory mechanism of photosystem stoichiometry were identified (Ozaki et al. 2007). These mutants are the disruptants of *slr1916*, *ccmK2*, *ctaEI*, *ctaCI*, *slr0645*, or *slr0249*. The two mutants (the *slr1916* and the *ccmK2* mutant) showed increasing PSI contents under high-light condition as in the case of the *pmgA* or the *sll1961* mutant. These findings may indicate that the ratio of PSI/PSII is regulated by rather complicated mechanisms, and there is a possibility that Sll1961 functions in a novel pathway, independent of the *pmgA* pathway.

Sll1961 has a helix-turn-helix domain on its N-terminus. To search for the targets of Sll1961, Fujimori et al. performed DNA microarray analysis using the *sll1961* mutant (Fujimori et al. 2005). Several genes were affected in their expression by the disruption of *sll1961*. The expression of *sll1773* was significantly induced in the *sll1961* mutant, while the expression of *slr0364*, *slr2076*, and *slr2057* were suppressed. The *sll1773* gene encodes a pirin-like protein called PirA (Hihara et al. 2004, 53083). It was reported that the expression of *pirA* (*sll1773*) increased under several stress conditions, so that the expression of *pirA* in the *sll1961* mutant might be increased due to severe stress condition caused by the combination of high-light and the disruption of the *sll1961* gene. The *slr0364* gene encodes a hypothetical protein, which has a Thr-rich region. Interestingly, the *slr0366* gene whose product has also a Thr-rich region is located in adjacent downstream of *slr0364*. The *slr2076* and *slr2057* genes encode a chaperonin (GroEL1) and a water channel protein (Aqpz),

respectively. These genes could be candidates for the targets of Sll1961, which might be a transcriptional regulator. In this chapter, I focus on these putative target genes, and studied the functions of these genes in photosystem stoichiometry in relation to *sll1961*.

Results

Transcriptional level of the putative target genes under the shift to high-light

Through the microarray analysis of the *sll1961* mutant, I picked up four genes, which were downregulated in the *sll1961* mutant, as candidates for the targets of transcriptional regulation by Sll1961. Then I estimated and compared the amounts of transcripts of those genes in the wild type and the *sll1961* mutant during the shift to high-light by the RT-PCR (Figure 7).

In the wild type, the transcript level of *sll1961* is constant irrespective of the light condition. On the other hand, the transcript of *slr0364* was almost undetectable under low-light, but immediately induced in 15 minutes upon the shift to high light condition, and continuously detected for 20 h. This pattern of expression of *slr0364* is consistent with the data from DNA microarray analysis performed by Hihara et al. (Hihara et al. 2001). The transcript level of *slr0366* and *slr2076* showed more or less similar expression to that of *slr0364*. As for slr2057, the expression was observed in low light condition as well as high light condition. However, the trascript level temporally decreased upon the shift to high light. In the *sll1961* mutant, I found that the transcriptional level of *slr0364*, *slr0366*, and *slr2057* were remarkably decreased than in the wild type. The transcriptional level of *slr2076* (*groEL1*) somewhat decreased from that in the wild type. It seems that the gene expression of *slr0364*, *slr0366*, and *slr2057* were affected by the disruption of *sll1961*, and these three genes would be candidates for the target of Sll1961. However, the expression patterns of these genes seemed to be similar in the wild type and the *sll1961* mutant, although the expression level was affected.

PSI/PSII ratio in the mutants that lack putative target genes of Sll1961

The *sll1961* mutant is unable to regulate photosystem stoichiometry under high-light condition. In order to know whether the target genes are involved in the regulation of photosystem stoichiometry, chlorophyll fluorescence spectra were determined at liquid nitrogen temperature (77 K) for the mutants of *slr0364*, *slr0366*, *slr0364/slr0366* (*double*), *slr2057*, and *sll1961*, and for the wild type. The fluorescence at 725 nm is predominantly emitted from PSI while that at 695 nm arises from PSII. Thus, the fluorescence intensity at 725 nm / fluorescence intensity at 695 nm (F_{725}/F_{695}) is a good index of PSI/PSII ratio. Figure 8 shows the relative PSI/PSII ratio of cells grown under high-light or low-light for 24 hours. Cells of every strain grown under low-light exhibited no significant difference in PSI/PSII. The *sll1961* mutant grown under high-light (200 μmol $m^{-2}s^{-1}$) had a higher level of PSI/PSII ratio, and the *slr0364*, *slr0366*, *double*, and *slr2057* mutants also showed slightly but significantly higher PSI/PSII ratio than in the wild type. The level of PSI/PSII ratio under high-light of the wild type, the *sll1961*, the *slr0364*, the *slr0366*, the *double*, and the *slr2057* mutants were 52%, 88%, 65%, 66%, 62%, and 57% of that under low-light, respectively. The result indicates that any single gene is not enough to explain the phenotype of the *sll1961* mutant during high light acclimation.

Chlorophyll content

Since chlorophyll molecules mainly bind to PSI in cyanobacteria, it is expected to detect higher level of chlorophyll content in the mutant that has increased level of PSI. Absorption spectra of whole cells of the wild type and the mutants suspended in BG-11 media was measured to calculate chlorophyll contents. In the *slr2057* mutant, I observed

significantly higher chlorophyll content than that of the wild type under high-light. The *slr0364* and the *slr0366* mutants showed higher chlorophyll contents at average level, but the difference was not statistically significant because of an experimental error. It implies that the increased level of the PSI/PSII ratio in the *slr2057* mutant is due to higher amount of PSI in the mutant (Figure 9).

Growth under photomixotrophic condition

It was reported that growth of some mutants, which are unable to decrease PSI content under high-light condition, is severely suppressed under photomixotrophic condition with 5 mM glucose and light (>50 μmol $m^{-2}s^{-1}$) (Hihara and Ikeuchi, 1997 and Ozaki et al., 2007). In the presence of 5 mM glucose under medium light (100 μmol $m^{-2}s^{-1}$), growth of the *slr2057* mutant was severely suppressed (Figure 10). It was suggested that the photomixotrophic condition might lead to some imbalance in cellular metabolism by the combination of CO_2 fixation via photosynthesis and the glucose uptake in the *pmgA* mutant (Hihara and Ikeuchi, 1997). Similar situation would be induced in the *slr2057* mutant.

Interaction between His- Sll1961 and the upstream region of putative target genes

To test the binding of Sll1961 to the upstream region of *slr0364, slr2057* and *slr2076*, I performed electrophoretic mobility shift assay (EMSA). Since *slr2076* was reported to be expressed as a polycistronic transcriptional unit with *slr2075* that encodes *groES* (Gratz et al., 1997), I used upstream region of *slr2075* gene instead of using intergenic region between *slr2075* and *slr2076*. In all experiment, I used DNA segments in size of 200 base pairs, which code upstream regions from the translational start point of indicated genes (see

Materials & Methods). As a first step, I expressed and purified His-Sll1961 protein with 6× His on its N-terminus using expression system of *Escherichia coli*. Using the DNA segment of 200 base pairs upstream region from translational start point of *slr0364*, I tested the DNA-Sll1961 interactions in the condition of 0~6.25 μM His-Sll1961 (Figure 11A). Only with 6.25 μM His-Sll1961 (lane 5), the promoter segment of *slr0364* showed band-shifts in two distinct electrophoretic mobilities. One of the DNA-protein complex is about the size of 300 bp as in DNA fragment (black arrowhead in Figure 11), the other corresponded to the size of 1033 bp~ as in DNA fragment (black arrow in Figure 11). I also detected the band shifts to these two DNA-protein complexes with promoter segments of *slr2057* and *slr2075* (Figure 11BC). These observations suggested that these three promoter segments showed same interaction manners with His-Sll1961, and these interactions are probably classified into at least two types. Then I estimated the composition of long retarded bands form the electromobility, and the large band (at black arrows) corresponded to a complex including as a probe and at least nine molecules of His-Sll1961. On the other hand, smaller band (at black arrowheads) was estimated as a complex including a probe and one to two molecule(s) of His-Sll1961. Nonamer is considered too large compared with the other GntR transcriptional regulators. It suggests that the large bands contains artificially formed aggregate, although the small bands seem to be in reasonable size compared with the other functional GntR transcriptional regulators. However, the addition of non-labeled competitor DNA together with Sll1961 protein did not suppress the bindings at the small band (Figure 11A~C, lane 6, 3, and 3, respectively). Competitor assay is generally used in order to confirm the sequence specificity in DNA-protein complexes. If a interaction is specific, a band-shift must be cancelled by the addition of unlabeled competitor DNA segments. I also tested the binding of His-Sll1961 to a

segment derived from pBluescript as a negative control sequence, and detected a large band same as the large band detected in EMSA with the other DNA segments(Figure 11D, size at black arrow). It may be an artificial interaction, but from these experiments, I could not observe the specific interaction between His-Sll1961 and any of the promoter segments. In other words, I could not judge whether the putative target genes are the direct target of Sll1961 or not. Nevertheless, the molecular size of the small bands (indicated in Figure 11 ABC, black arrowheads) is likely to be exact complex of His-Sll1961 and the promoter segments of *slr0364*, *slr2057*, or *slr2075*, the possibility that Sll1961 specifically interact with the promoter segments of the putative target genes in cirtain experimental condition, in which Sll1961 is caused an increase in its activity .

Interactions between putative promoter segments and crude proteins from Synechocystis.

Previous studies strongly suggested that the role of Sll1961 is reduction of high-light stress in cyanobacteria (Fujimori et al., 2005). If Sll1961 works as a transcriptional regulator of *slr0364, slr2057 or slr2076* under high-light, (i) the binding manner of Sll1961 must be different between under high-light and under low-light in the wild type cells, (ii) the band shift must be different between in the wild type and in the *sll1961* mutant. Then I performed EMSA with cyanobacterial protein extracts from cells of the both wild type and the *sll1961* mutant grown under high-light or low-light. Through the EMSA with DNA segment of pBluescript, the intensity of the band corresponding to the free probe (Figure 12D, white arrowhead) decreased exclusively by the addition of crude protein extract from the *sll1961* mutant grown under high-light. It implies that the disruption of *sll1961* causes severe stress under high-light, and some proteins are denatured form in vivo. Then the protein extract from

the *sll1961* mutant grown under high-light might easily form non-specific interactions with DNA.

Through the EMSA with promoter segment of *slr2057*, an interaction was observed with protein extracts from the wild type grown under high-light (Figure 12 B, at black arrowhead). This complex shifted to the size calculated as one to two Sll1961 molecules per one *slr2057* probe, and this composition coincides with the small complex (see Figure 11) observed in the EMSA with His-Sll1961. Moreover, it was observed that the free promoter segment of *slr0364* and *slr2075* were shifted with the addition of protein extract from high-light acclimated wild type cells (Figure 12A lane 3 & C lane 2). The size of the complex with the segment of *slr0364* was suspected to be same size with the complex contained segment of *slr2057*, while the eretrophoretic mobility the segment of *slr2075* were not detected.

EMSA with protein extracts from the wild type and the *sll1961* mutant grown under low-light were examined. As a result, the band patterns of each three promoter segment were not different between protein extracts from the wild type and the *sll1961*mutant (Figure 12 A~C). So I speculated that some transcription factor other than Sll1961 functions under low-light condition on the promoter site of *slr0364, slr2057* and *slr2075*.

From EMSA with cyanobal crude proteins, it was indicated that the *sll1961* mutant is exposed to severe stress condition under high-light. It was also implied that there remain the possibility that Sll1961 is directly regulates *slr0364*, and *slr2057*, (or slr*2075*) under high-light , because of the formation of complex which is in same molecular size with the complex that may contain Sll1961 and the DNA segments. To judge whether Sll1961 is a transcriptional regulator and regulate the putative target genes, further study must be done.

Discussions

From the result in RT-PCR, it is shown that *sll1961* is constitutively transcribed under low-light as well as under high-light (Figure 7), although the *sll1961* mutant shows specific phenotype not under low-light condition but under high-light condition (Fujimori et al., 2005). This might indicate that *sll1961* is regulated at post-transcriptional level, and somehow functions after the shift to high-light.

Although the *slr0364* and the *slr0366* mutant showed weak defect in the regulatio of PSI/PSII ratio upon high-light acclimation (Figure 8), these two mutants showed higher but non-significant level of chlorophylls per cell basis than the wild type under high-light (Figure 8). I cannot conclusively say that these two genes are involved in the regulation of photosystem stoichiometry but a part of the phenomenon might be regulated by these genes.

The *slr2057* mutant showed high PSI/PSII ratio under high-light, and also showed higher chlorophyll content per cell basis under high-light (Figure 8, 9). The chlorophyll content is a good index of PSI amount since nearly 90% of the chlorophyll molecules are associated with PSI complexes in cyanobacteria. It indicates that high PSI abundance causes the imbalance of PSI/PSII under high-light in this mutant. The slr2057 mutant also showed the suppression of photomixotrophic growth. These phenotypes are consistent with the mutants that classified into the group I mutants by Ozaki et al. (2007). The addition of glucose under light illumination probably lead excess influx of electrons into the photosynthetic chain from the respiration in cyanobacteria, in which photosynthesis and respiration shares some electron transfer components. In this way, a defect in the decrease of PSI content under high-light conditions would lead to the growth sensitivity under this condition. The precise mechanism of this sensitivity is currently unknown yet. As *slr2057* codes water channel

(aquaporin z), people may wonder how the disruption of aquaporin affects PSI abundance under high-light. One explanation is the effect of CO_2 concentration on PSI/PSII. It is already reported that CO_2 passes through Aquaporin in *Synechocystis* (Tchernov et al., 2002). The decrease of CO_2 concentration in cyanobacterial cell is known to increase PSI/PSII (Murakami et al., 1997). Under high-light condition, high photosynthetic rate lead to the low cellular CO_2 concentration. We can think just the other way around. The lowered cellular concentration of CO_2 due to the defect in aquaporin may induce high PSI abundance leading to the high PSI/PSII ratio. In this context, it should be noted that the insertion mutant of *ccmK2* gene, whose product is involved in the CO_2 concentrating mechanism in cyanobacteria, also showed defect in the regulation of photosystem stoichiometry (Ozaki et al., 2007). It was shown that the expression of many genes were regulated by the balance between photon flux density and concentration of CO_2 (Bdger et al., 2003). It is reasonable to assume that the CO_2 condition affects the response to light environment either metabolically or through some signal transduction pathways.

In the EMSA with purified His-Sll1961 (Figure 11), I observed two interactions between His-Sll1961 and promoter segments of *slr0364*, *slr2057*, and *slr0366*. One of the interactions (large complex) is estimated to contain nonameric His-Sll1961. Therefore, it might be a non-specific interaction with aggregated His-Sll1961 and the promoter segments. This aggregation seems to be a result of high concentration of His-Sll196 (6.25 μM) in the reaction mixture, but I couldn't detect any band shift in the lower concentration of His-Sll1961 (Figure 12A). This result shows His-Sll1961 used in this experiments has relatively low binding ability, while transcription factors are generally known to be active only in nM order. It suggest that we must pay attention to the specificity of DNA –Sll1961 interactions.

The other interaction (small complex) ,obtained from the EMSA with His-Sll1961, is estimated to contain one to two molecule(s) of His-Sll1961. This DNA-Protein complex is consistent to the size of GntR transcriptional regulator formerly reported. Sll1961 is classified into GntR type transcriptional regulator, because of its herix-turn-herix construction. GntR transcriptional regulators share a similar N-terminal DNA-binding (D-b) domain, and have divergentt C-terminal effector-binding and oligomerization (E-b/O) domain (Rigali et al., 2002). Some of the GntR transcriptional regulators are known to require oligomerizations (usually dimerization) or an effector binding at their E-b/O domain for their binding abilities(Sung et al., 1991, Ko et al., 1999). Therefore, the small complexm, including His-Sll1961 and the promoter segments, is possibly in natural form like the other GntR transcriptional regulators. From the results that competitor DNA of each promoter segment did not cancelled the interaction in the small complex, it may be suggested that the sequence specificity was not observed in the interactions between His-Sll1961 and the promoter segments of *slr0364*, *slr2057*, or *slr2075*. It indicates that His-Sll1961 has an ability to associate with some DNA sequences, but sequence-specific bindings of His-Sll1961 were not observed in this experimental conditions. As mentioned above, some GntR transcriptional regulators need effectors to be activated, so any effectors may be required for activation of His-Sll1961.

Materials & methods

Strains and growth conditions

A glucose-tolerant WT strain of *Synechocystis* sp. PCC 6803 and the mutant strains were grown at 30°C in BG-11 medium (Rippka et al., 1979) with 10 mM N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid-KOH (pH 8.0). Unless stated otherwise, cultures were grown under continuous illumination provided by fluorescent lamps at 20 mmol photons $m^{-2}s^{-1}$. Cells were grown in test tubes (3 cm in diameter) and bubbled with air. Solid medium was supplemented with 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate. high-light shift experiments were performed by transferring cells at the exponential growth phase from low-light (20 μmol photons $m^{-2}s^{-1}$) to high-light conditions (200 μmol photons $m^{-2}s^{-1}$).

Measurement of chlorophyll fluorescence emission spectra at 77K

Low temperature fluorescence emission spectra at liquid nitrogen temperature (77K) were recorded using a custom-made apparatus (Sonoike and Terashima, 1994). Cell suspensions at a chlorophyll concentration of 5 mg chlorophyll ml^{-1} in BG-11 medium were injected into brazen sample holders frozen by immersing them into liquid nitrogen. The cell suspensions were excited by blue light passing through a filter (CS 4-96, Corning Inc., USA) or green light passing through a filter. Before the measurement, cells were incubated in darkness for 10in at room temperature to equilibrate the redox state of photosynthesis electron transfer chain. Under this condition, the intensity ratio of PSI fluorescence at 725 nm and PSII fluorescence at 695 nm gives information on the relative ratio of photosystem contents. Chlorophyll *a* concentrations were determined after extraction with 100% methanol (Grimme and Boardman, 1972).

Measurement of pigment content

In vivo absorption spectra of whole cells of the wild type and mutants suspended in BG-11 medium were measured at room temperature using a spectrophotometer (Model 356, Hitachi, Japan) with a cuvette place just in front of a photomultiplier to minimize the effect of scattering on absorption spectra. Concentration of chlorophyll was calculated by the equations of Arnon et al. (1974). Chlorophyll content of cells was normalized at OD_{750} determined by a spectrophotometer (Model 356, Hitachi, Japan).

RT-PCR and real-time RT-PCR analysis

Total RNA solutions were isolated by hot-phenol method (Muramatsu and Hihara, 2003). Quantitative RT-PCR was performed with RNA PCR Kit (AMV ver.3.0, Takara, Japan).

Overexpression and Purification of His-Sll1961

The plasmid pET1961 created for expression of a fusion protein of Sll1961 with an N-terminal His tag was kindly gifted from Hihara laboratory in Saitama University. Overnight grown culture of strain BL21 harboring pET1961 was diluted 1:100 with TB medium and returned to the shaker–incubator at 37°C. At mid-log phase (OD_{600}=0.5-0.6), isopropyl-β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM and cells were cultured for additional 8h at 25℃. Cells were harvested by centrifugation at 3000 rpm for 10 min, then stored at –80℃. Cells were resuspended in Lysis buffer (50 mM phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole), and disrupted by sonication for 10 s×5 times. The lysate was centrifuged at 18800×g for 15 min, the supernatant was collected as soluble protein fraction. His-Sll1961 was purified by Ni-NTA Superflow (Qiagen). The soluble protein fraction was diluted to 2 μg protein/mL by Lysis buffer, 1/10 volume of Ni-NTA Superflow was added and

incubated on a rotary shaker for 30 min. The resin was gathered by centrifugation at 3000 rpm for 1 min, washed twice with Wash buffer (50 mM phosphate [pH 8.0], 1 M NaCl, 20 mM imidazole) and eluted 3 times by Elution buffer (50 mM phosphate [pH 8.0], 300 mM NaCl, 150 mM imidazole). The eluted protein fraction was desalted by alternating between concentration and dilution with centriprep YM-10 column (Millipore) and with phosphate buffer [pH 8.0]. Finally, desalted protein fraction was concentrated with Microcon YM-10 column (Millipore).

Gel Mobility Shift Assay

For preparation of the proves and the specific competitor DNA fragments for gel mobility shift assays, DNA fragments corresponding to the promoter region of each gene were obtained by PCR amplification. The 3' -end of the DNA fragment was labeled with DIG-ddUTP by the terminal transferase according to the manufacturer' s instructions (DIG gel shift kit; Roche). Purified His-Sll1961 protein was incubated with 30 fmol DIG-labeled DNA fragment in a 10 μl reaction mixture containing 0.5 μg of polyd[I · C], 0.05 μg of poly-L-lysine, 20 mM Hepes-KOH, pH7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 0.2% (w/v) Tween 20, and 30 mM KCl. After incubation, 2.5 μl of gel loading buffer consisting of 60% (v/v) of 1× Tris borate-EDTA and 40% (v/v) glycerol was added to the reaction mixture. Samples were then applied onto a 8% polyacrylamide gel and subjected to electrophoresis at 80 V for 6 h at 4℃. DNA and protein were transferred to a nylon membrane (Hybond N+; Amersham Bioscience) by electro-blotting method and fixed by UV cross-link at 7 mJ/cm^2. DIG-chemiluminescent detection was performed according to the manufacturer's protocol (Roche).

Preparation of protein extract from cyanobacteria

Low-light- or high-light-exposed *Synechocystis* cells in exponential phase was harvested by 3000 rpm for 10 min. The pellet was resuspended in 1ml of extraction buffer,. The cell suspension was mixed with an approximately 500 ml volume of zircon beads and disrupted by vigorous vortexing three times for 2 min followed by cooling on ice for 1 min. After removal of the zircon beads, 200 ml of 5M NaCl was added to the crude extract and centrifuged at 17,400×g for 20 min. Proteins were precipitated by adding 0.4 g of solid $(NH_4)_2SO_4$ to 1ml of extract. After resuspension in 1ml of extraction buffer, the extract was dialyzed twice for 12 h against 1 l of the extraction buffer. All manipulations were carried out at 4°C.

Measurement of cell diameter

Cyanobacterial cells were harvested, then pictured by phase-contrast microscopy (Axioimager; Zuais)

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Figures

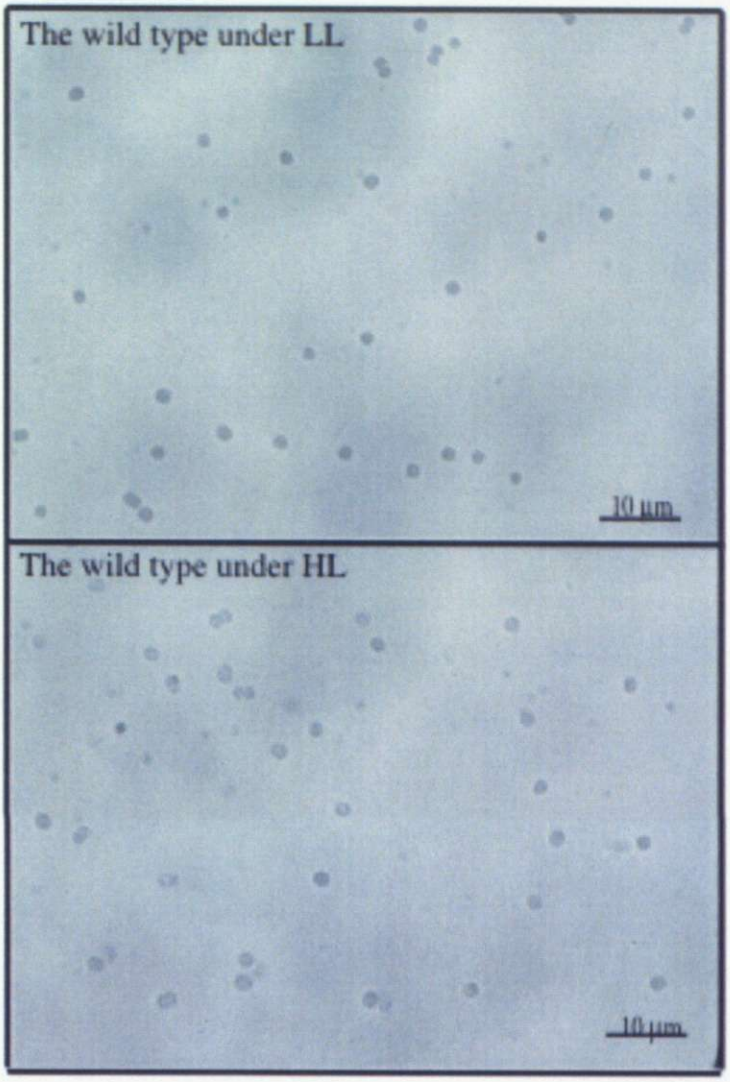

Figure 1. The cell morphology of the wild type under LL and HL. The wild type grown under LL was shifted to HL. At 12 h after the shift to HL, cells were collected, then pictured under bright light by microscope.

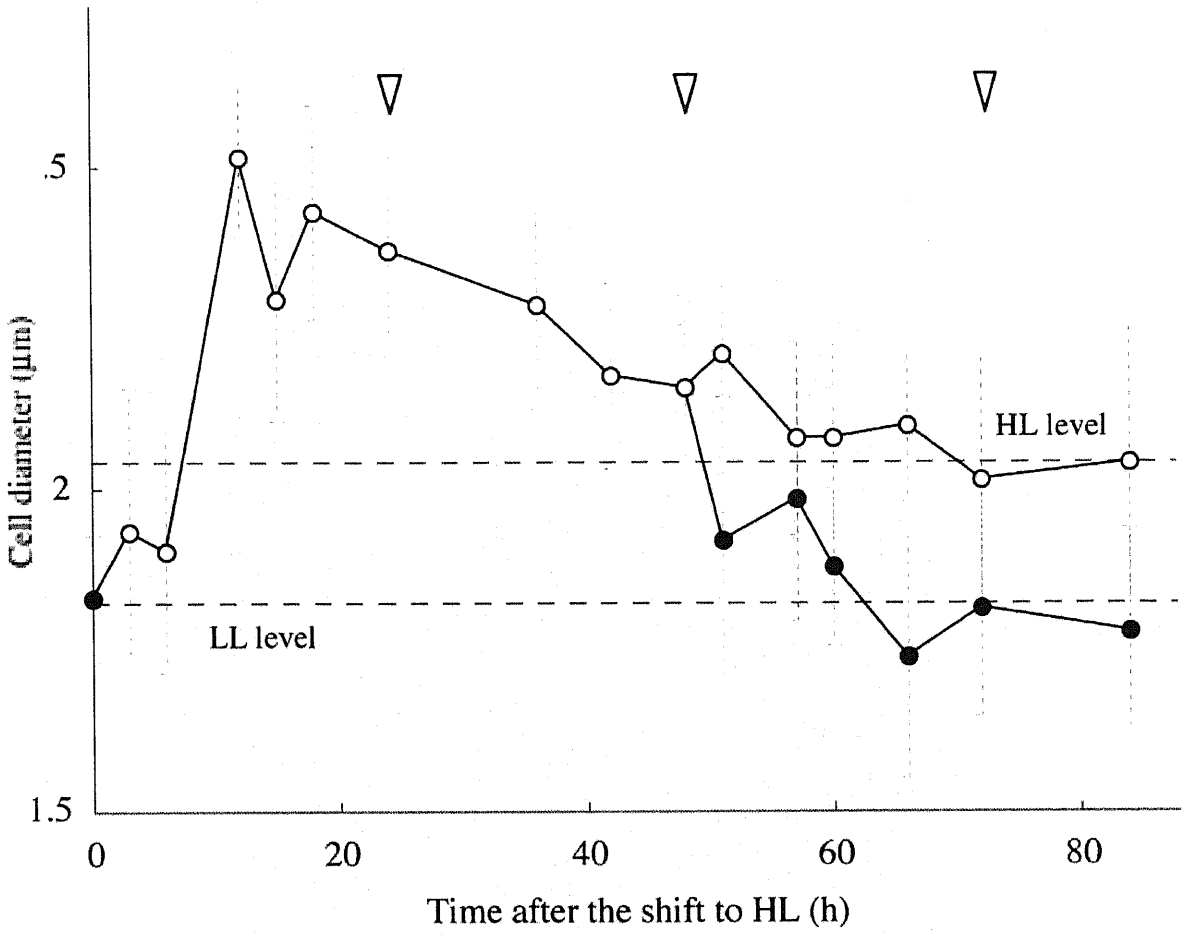

Figure 2. The change in cell size after the shift to HL. The wild type grown under LL were shifted to HL at time 0. At each time point, cells were collected and pictured by phase-contrast microscope. To prevent the self-shading, cultures were diluted every 24 h (indicated in arrowhead). The cells in LL condition are shown in closed circle, and cells in HL condition are shown in open circle. Each value is the average of twenty cells, and error bars indicate the SD. The result is representing two independent experiments.

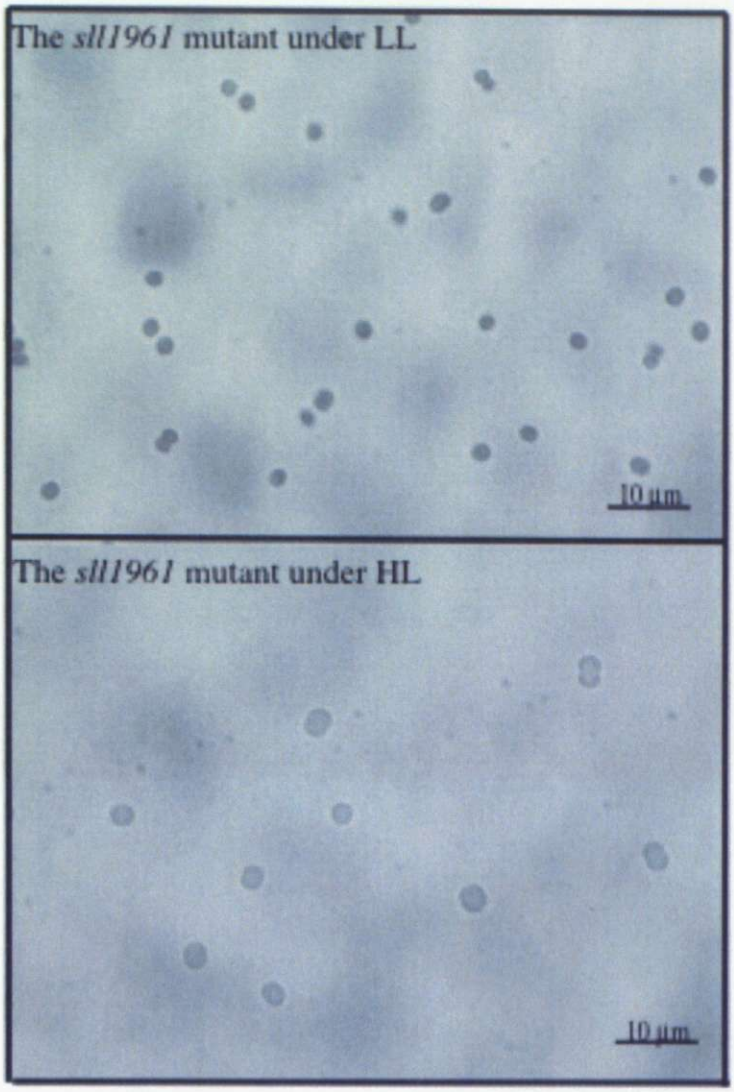

Figure 3. The cell morphology of the *sll1961* mutant under LL and HL. The *sll1961* mutant grown under LL was shifted to HL. At 12 h after the shift to HL, cells were collected, then pictured under bright light by microscope.

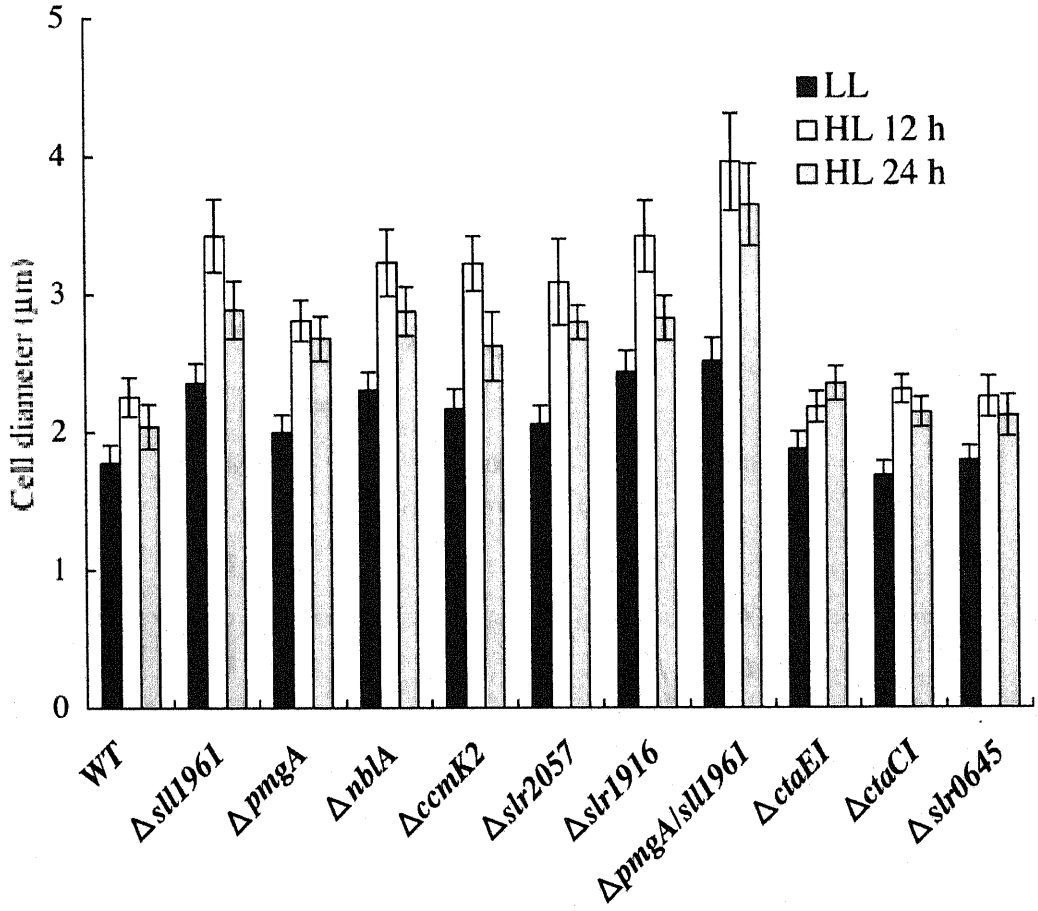

Figure 4. The change in cell size of the photosystem stoichiometry mutants and the wild type. Cells of each strains were grown under LL (black bars), and shifted to HL. After the HL illumination for 12 h (white bars) or 24h (graish bars), cells were collected and pictured by phase-contrast microscope. Each value is the average of twenty cells, and error bars indicate the SD. Same results were obtaine in three indipendent experiments.

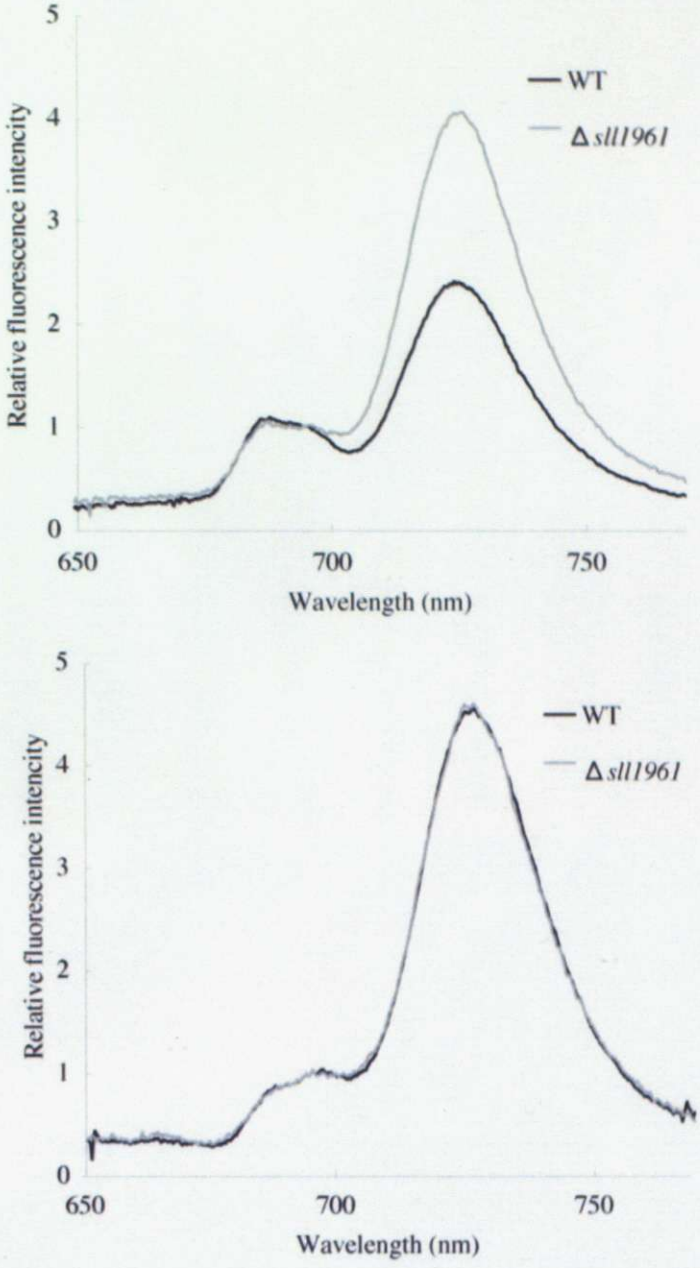

Figure 5A. Chlrophyll fluorescence emission spectra at liquid nitrogen temperature. The wild type (black line) and the *sll1961* mutant(gray line) were grown under HL (upper column) and LL(lower column) for 24 h.

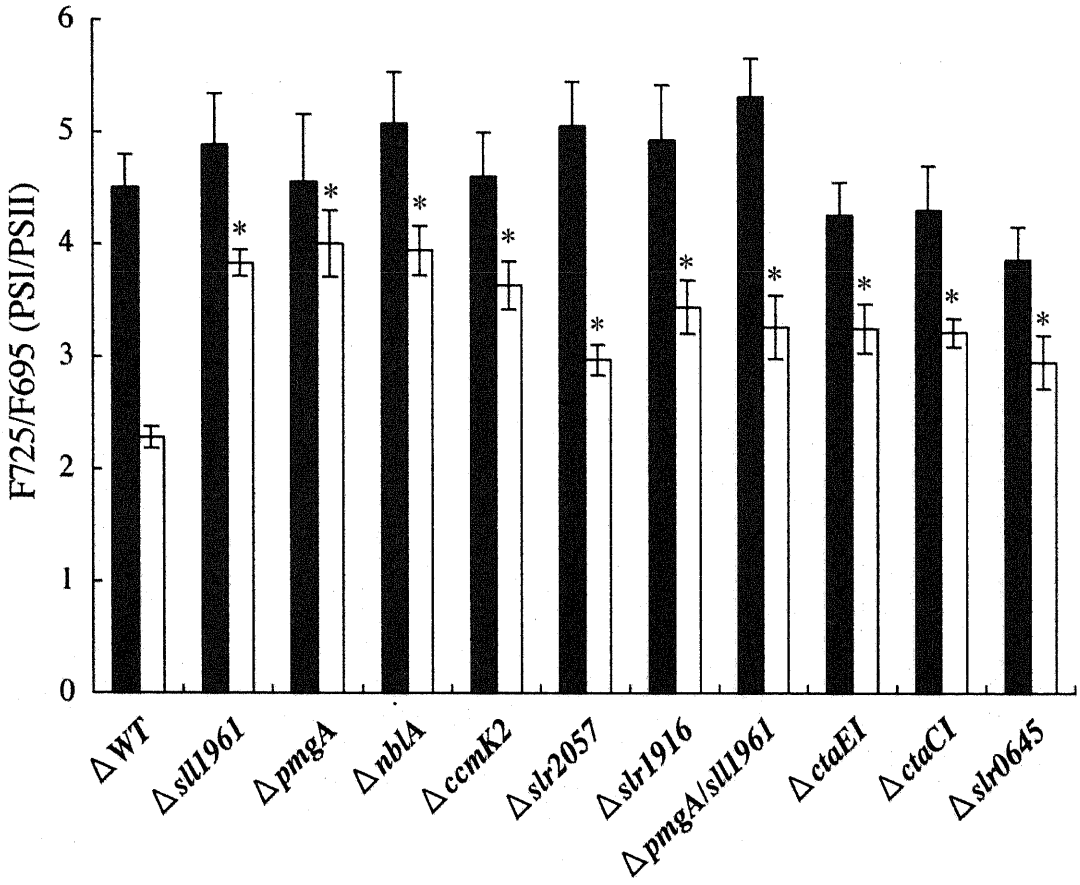

Figure 5B. The F_{725}/F_{695} in the photosystem stoichiometry mutants and the wild type. The wild type and the mutants were grown under LL (black bars) and HL (white bars) for 24 h. The ratio of F_{725}/F_{695} was determined by measuring chlorophyll fluorescence emission spectra at liquid nitrogen temperature (represented in Figure 5A). Each balue is the mean of three samples, and error bars indicate the SD. Asterisks indicate significant differences from the values of wild type (Student's t-test; $P<0.05$).

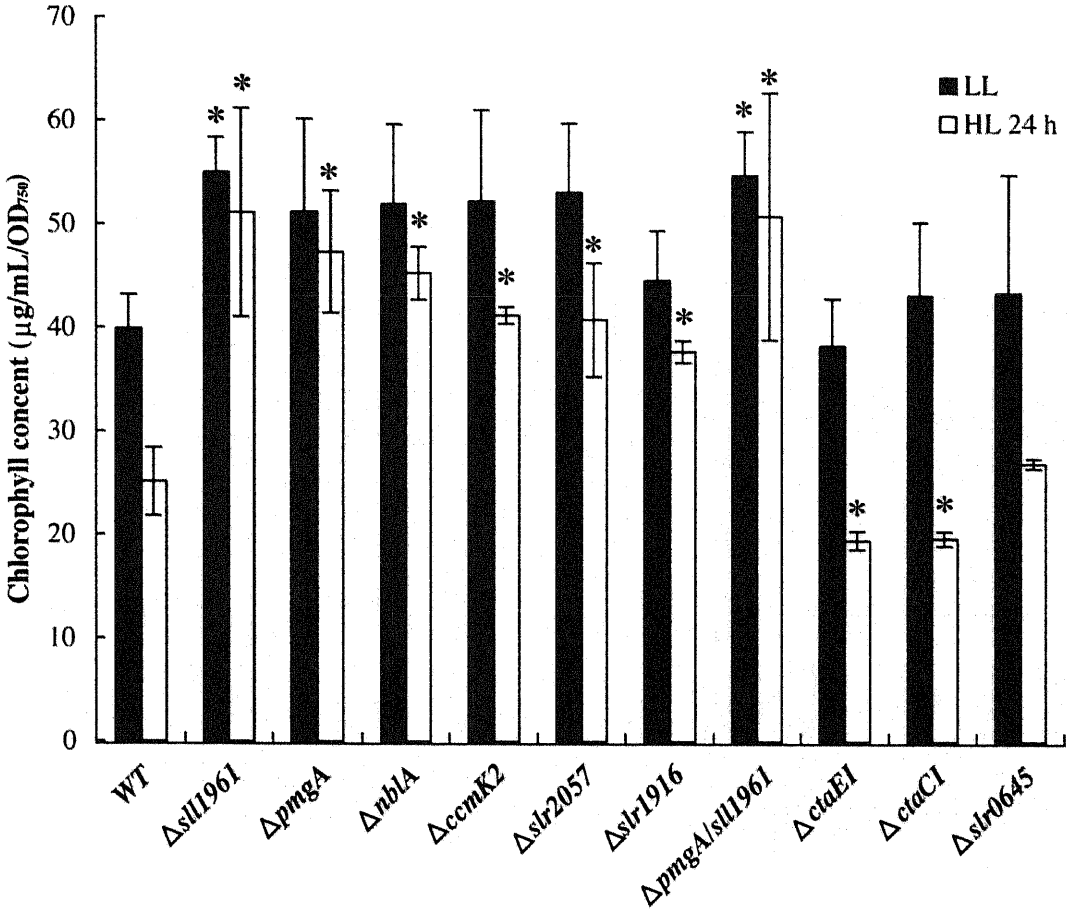

Figure 6. The chlorophyll contets in the wild type and the photosystem stoichiometry mutants. The wild type and the mutants were grown under LL (black bars) and HL (white bars) for 24 h. The concentrations of chlorophyll were calculated by the equations of Arnon et al. (1974) from the absorption spectra of the cells. The chlorophyll contents were normalized at OD_{750}. Each balue is the mean of three samples, and error bars indicate the SD. Asterisks indicate significant differences from the values of wild type (Student's t-test; $P<0.05$).

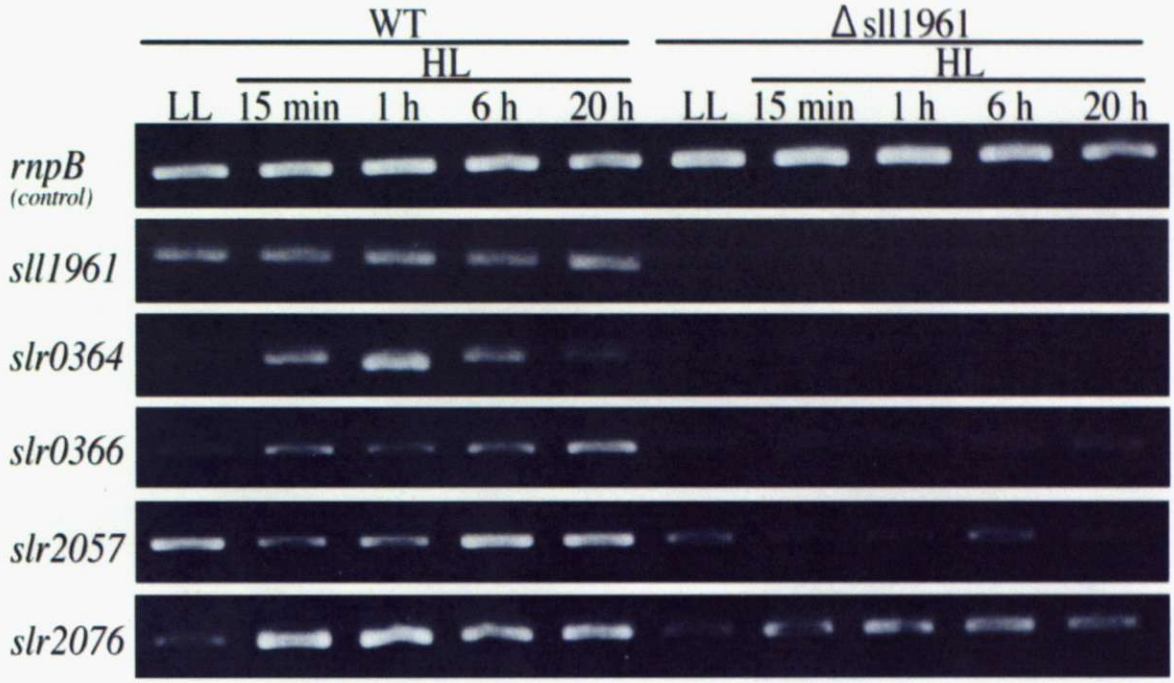

Figure 7. Transcriptional level of the putative target genes and the *sll1961*. The wild type and the *sll1961* mutant grown under LL were shifted to HL (time 0), then harvested at indicated time point after the shift to HL. The extracted total RNA were used for the semiquantitative RT-PCR analysis. Names for the primers used in the reactions are indicated at the left of clumns. Typical results from three independent experiments were represented.

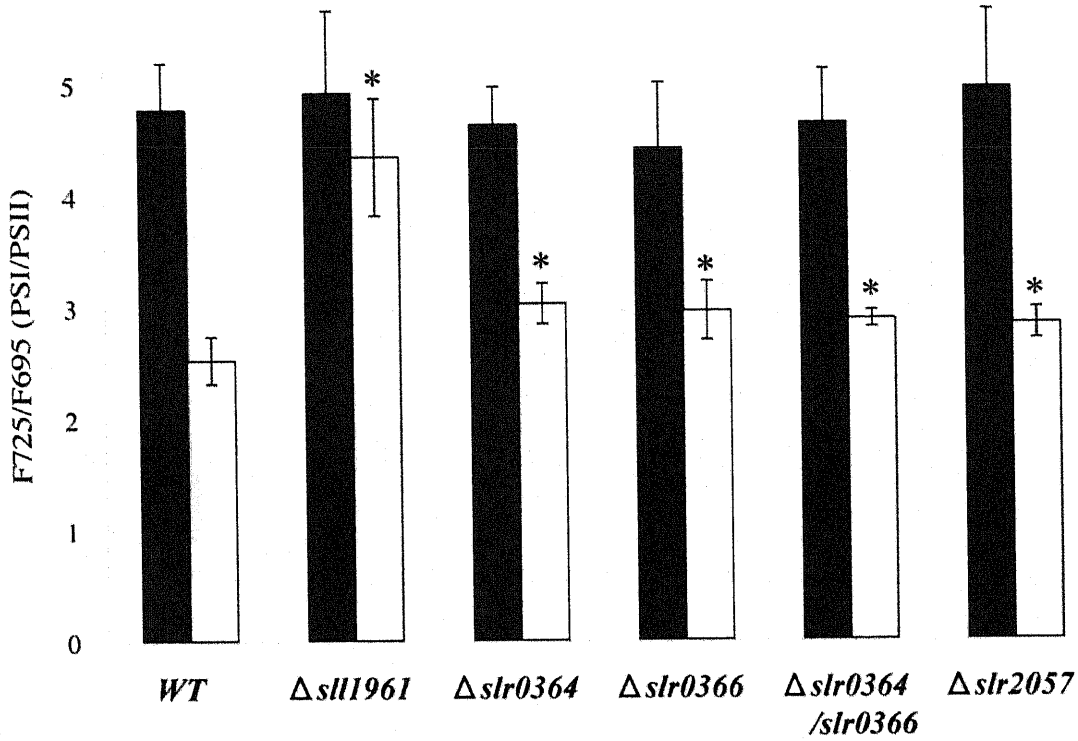

Figure 8. The F_{725}/F_{695} in the putative target mutans, the *sll1961* mutant, and the wild type. The wild type and the mutants were grown under LL (black bars) and HL (white bars) for 24 h. Each value is the mean of three samples, and error bars indicate the SD. Asterisks indicate significant differences from the values of wild type (Student's t-test; $P<0.05$).

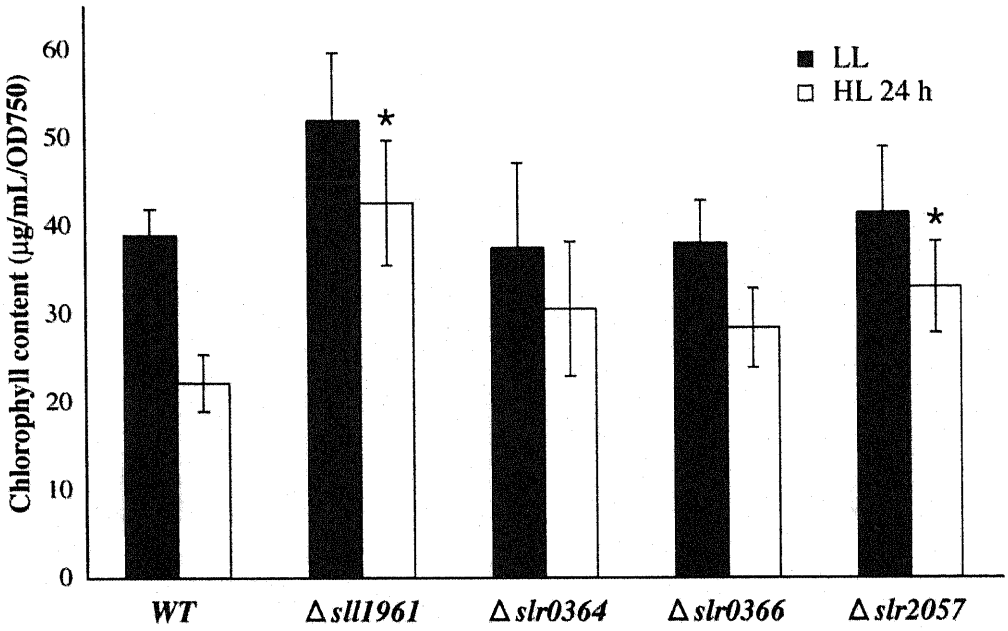

Figure 9. The chlorophyll contents in the putative target mutants, the *sll1961* mutant, and the wild type. The wild type and the mutants were grown under LL (black bars) and HL (white bars) for 24 h. Each value is the mean of three samples, and error bars indicate the SD. Asterisks indicate significant differences from the values of wild type (Student's t-test; $P<0.05$).

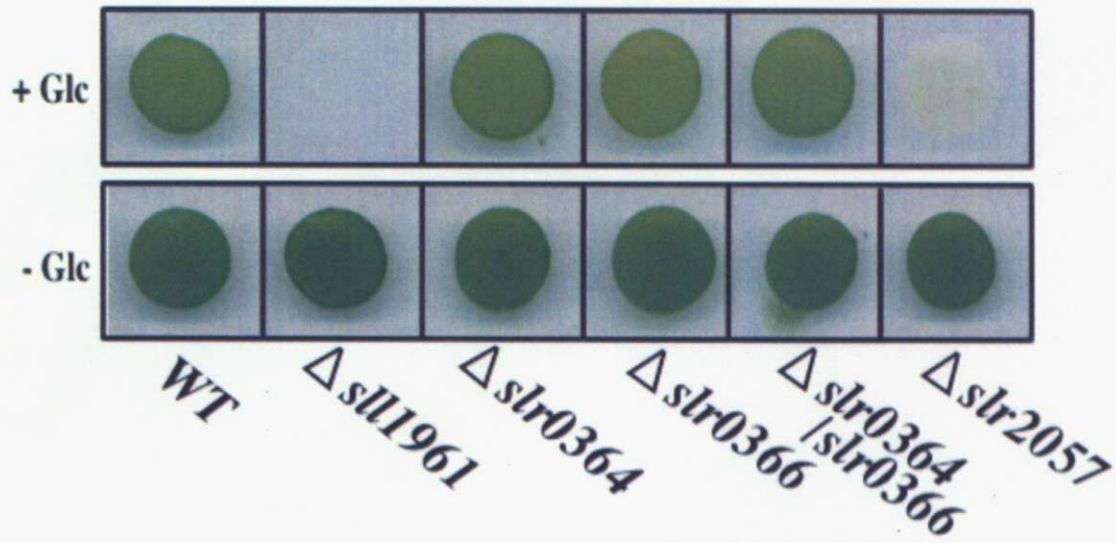

Figure 10. Growth properties of the wild type and the mutants under photomixotrophic condition. Liquid cultured cells (OD_{750}=0.1)grown under low-light condition were spotted on solid BG-11 medium. The cells were then grown for 3 days in the presence or absence of 5 mM glucose (Glc) under 100 $\mu molm^{-2}s^{-1}$)

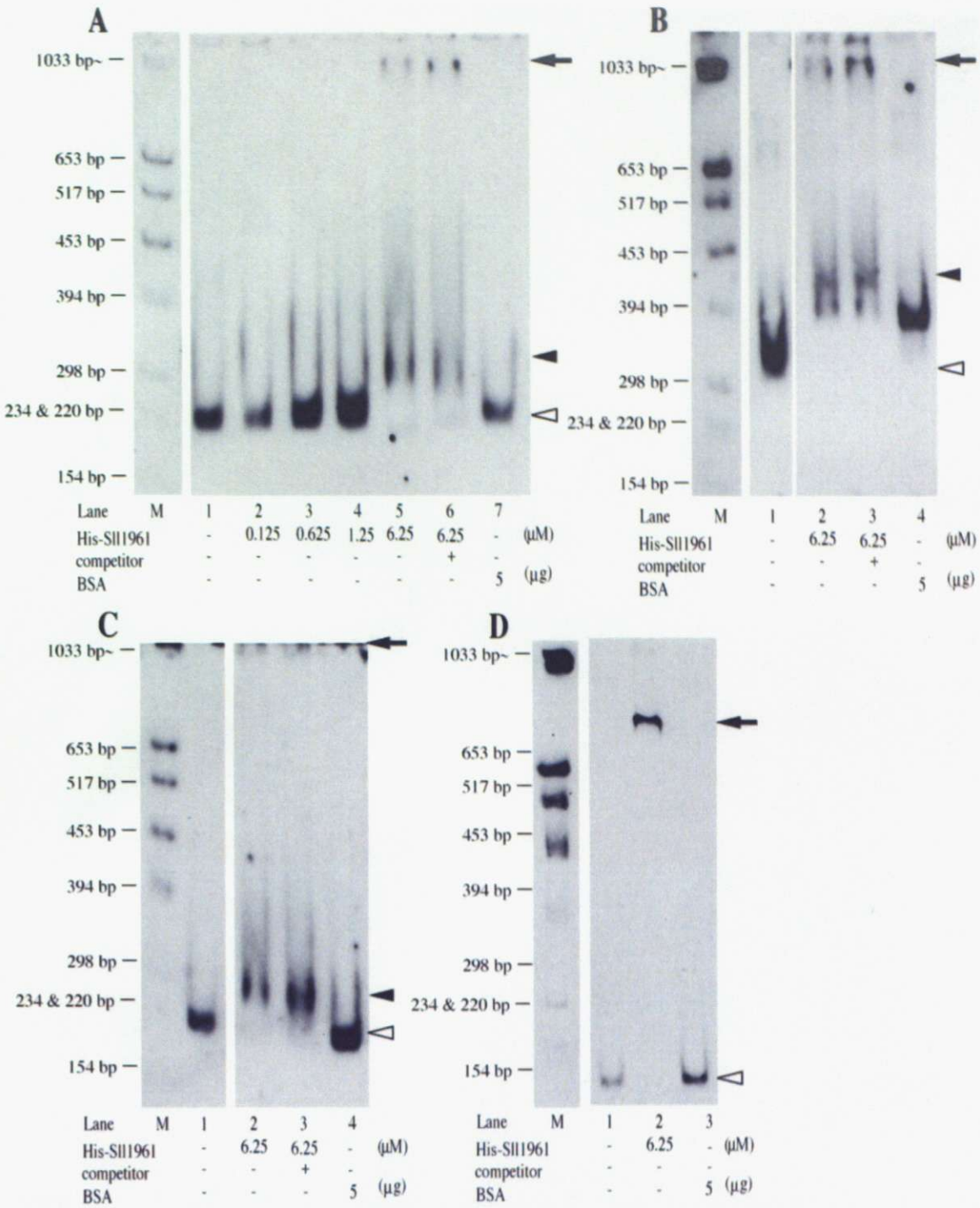

Figure 11. Gel mobility shift assay with the promoter segments of *slr0364* (A), *slr2057* (B) *slr2075* (C) or a DNA segment derived from pBluescript (D). The DIG-labeled promoter segments of each genes were incubated with ~6.25 μM His-Sll1961 in the presence of reagents (shown at the bottom of each column) for 15 min at 25℃. "M" indicate molecular weight markers. His-Sll1961 was obtained by the purification process shown in Materials & methods. 125-fold excess amounts of the non-labeled promoter segment were added as a competitor (indicated as Competitor). The 5 μg of Bovine serumn albumin were added as negative control (indicated as BSA) White arrowhead indicate the free probes, the black arrowhead indicate the small complex (see result), and .the black arrow indicate the large complex mentioned in Result.

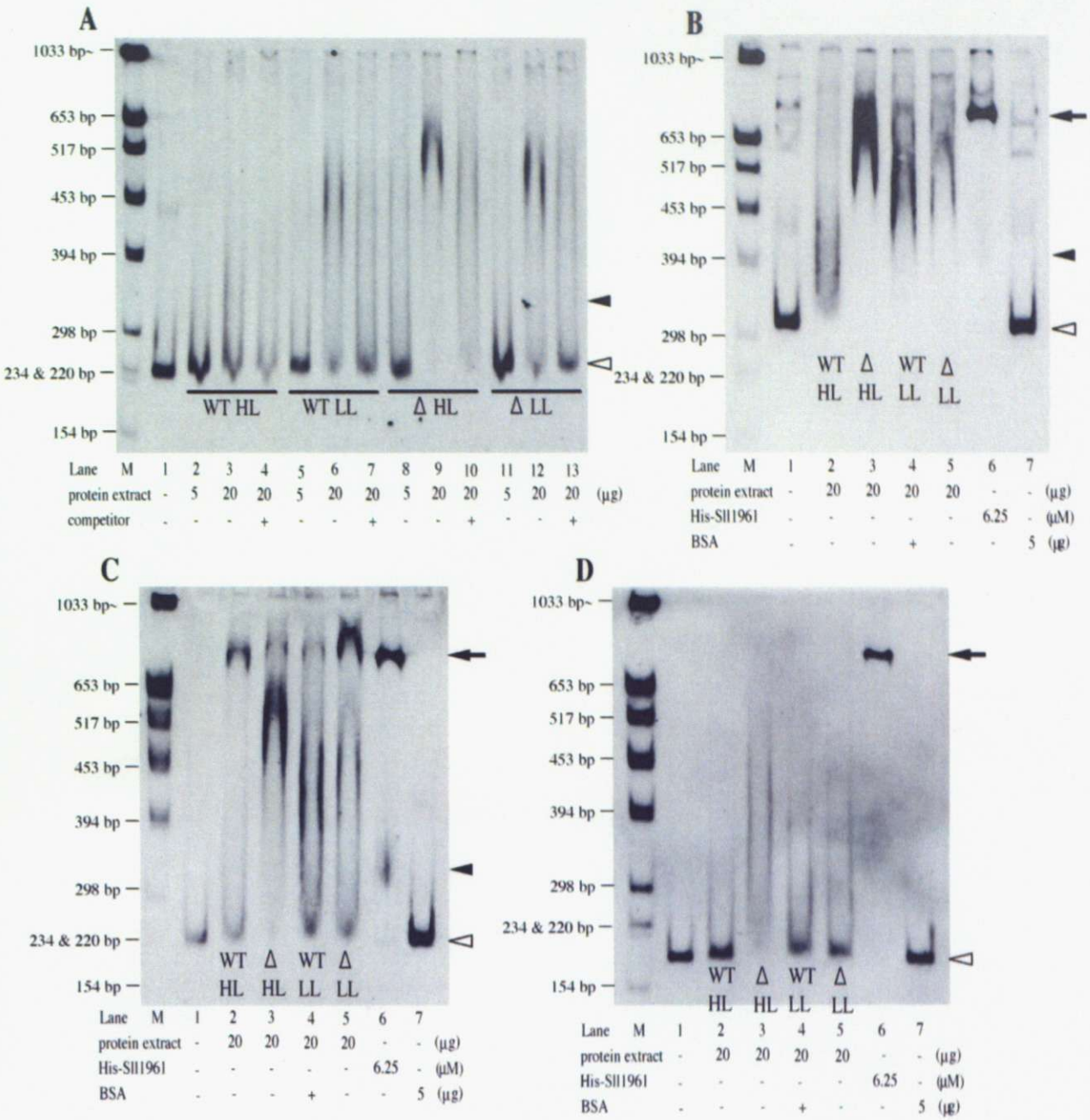

Figure 12. Gel mobility shift assay with the promoter segments and the crude protein extracts from cyanobacterial cells. The DIG-labeled promoter segment of *slr0364* (A), *slr2057* (B) *slr2075* (C) or a DNA segment of pBluescript (D) were incubated with cyanobacterial protein extracts derived from the wild type or the *sll1961* mutant, which were grown under LL or HL for 12 h in the presence of reagents (shown at the bottom of each column) for 15 min at 25℃. "M" indicate molecular weight markers. Cyanobacteiral protein extracts were obtained through the preperatioin shown in Materials & methods. 250-fold excess amounts of the non-labeled promoter segment were added as a competitor (indicated as Competitor). The 5 μg of BSA (bovine serumn albumin) were added as negative control (indicated as BSA).