Since chlorophyll fluorescence reflects the redox state of photosynthetic electron transport chain, monitoring of chlorophyll fluorescence has been successfully applied for the screening of photosynthesis-related genes. Here we report that the mutants having a defect in the regulation of photosystem stoichiometry could be identified through the simple comparison of the induction kinetics of chlorophyll fluorescence. We made a library containing 500 mutants in the cyanobacterium *Synechocystis* sp. PCC 6803 with transposon-mediated gene disruption, and the mutants were used for the measurement of chlorophyll fluorescence kinetics for 45 s. We picked up two genes, *pmgA* and *sll1961*, which are involved in the modulation of photosystem stoichiometry. The disruptants of the two genes share common characteristics in their fluorescence kinetics, and we searched for mutants that showed such characteristics. Out of six mutants identified so far, five showed a different photosystem stoichiometry under high-light conditions. Thus, categorization based on the similarity of fluorescence kinetics is an excellent way to identify the function of genes.

**Keywords:** Chlorophyll fluorescence — Cyanobacteria — Gene function — Mutant library — Photosystem stoichiometry.

Abbreviation: ORF, open reading frame.

### Introduction

The complete sequences of nearly 300 genomes have become available, with another 750 projects underway. Although information on the sequence of genomes provides the foundation to analyze genes on the genomes, the sequence information itself is not sufficient to reveal the function of each gene. For that purpose, phenotype analysis of mutants is necessary. The large-scale analysis of mutant libraries should potentially facilitate the comprehensive identification of the function of genes in a wide range of biological processes. Generating mutant libraries through random whole-genome transposon insertion mutagenesis followed by sequence-based identification of insertion sites has been used for a wide variety of microbes such as *Neisseria meningitidis* (Geoffroy et al. 2003), *Mycoplasma* (Hutchison et al. 1999), *Pseudomonas aeruginosa* (Jacobs et al. 2003), *Helicobacter pylori* (Salama et al. 2004) and *Saccharomyces cerevisiae* (Scherens and Goffeau 2004). Screening of the mutant libraries allows the characterization of many known and unknown genes by looking at phenotypes such as sporulation (Ross-Macdonald et al. 1999), growth in high-salt medium (de Jesus Ferreira et al. 2001) or ethanol tolerance (Takahashi et al. 2001).

For plants and other photosynthetic organisms, chlorophyll fluorescence has often been used for screening. The yield of chlorophyll fluorescence from photosynthetic organisms that have been dark adapted for some time shows complex kinetic changes upon actinic illumination. This kinetics of chlorophyll fluorescence are called the ‘Kautsky effect’, which reflects the change in the condition of photosynthesis that is the most important metabolic pathway in plants (Govindjee 1995). Use of chlorophyll fluorescence is advantageous for large-scale screening, since it can be easily monitored non-destructively using a charge-coupled device (CCD) camera. For example, mutants with defects in xanthophyll cycle activity were isolated by the analysis of digital video imaging of chlorophyll fluorescence in *Chlamydomonas reinhardtii* (Niyogi et al. 1997) or in *Arabidopsis thaliana* (Niyogi et al. 1998, Li et al. 2000). Similar imaging of chlorophyll fluorescence was used for isolation of mutants with modified NAD(P)H dehydrogenase activity in *A. thaliana* (Hashimoto et al. 2000).
actinic light was used as the criterion for the activity of NAD(P)H dehydrogenase. Although these methods enable us to search for mutants that have defects in a specific function, they cannot be applied to the screening of the mutants whose phenotype is not clearly established.

We propose a new type of screening in this study. The method is simply based on the similarity of fluorescence kinetics among the mutants and does not require the theoretical relationship between the kinetics of fluorescence and the physiological condition of the mutants. Recently, we isolated several mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 that showed induction kinetics of chlorophyll fluorescence different from those of the wild type. Among the mutants, the sll1961 mutant exhibited fluorescence kinetics similar to those of the pmgA mutant that has a defect in the regulation of photosystem stoichiometry (Fujimori et al. 2005). Indeed, the detailed characterization of the sll1961 mutant revealed that this mutant could not regulate photosystem stoichiometry. Thus, the results open the way for a method to identify a mutant with a defect in the regulation of photosystem stoichiometry by simply monitoring the kinetics of chlorophyll fluorescence. In this study, we made a library of cyanobacterial mutants using transposon-mediated mutagenesis, and screened the library using the similarity of the fluorescence kinetics to one another. The results clearly indicated the practical effectiveness of the method focusing on the fluorescence kinetics pattern to identify the mutant with modified photosystem stoichiometry.

**Results**

**Construction of a *Synechocystis* mutant library**

A total of 106 cosmid clones used for the whole-genome sequencing of *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996), which cover 89% of the open reading frames (ORFs) in the genome, were used for the mutagenesis. Each cosmid clone was mutagenized in vitro with the transposon and transformed into *Escherichia coli* cells to pick up individual mutated cosmid clones. By sequencing the border regions between the transposon and *Synechocystis* genomic DNA using a transposon-specific DNA primer, 1,092 cosmid clones with disruption at different ORFs were selected. They were then used for generating inseritional mutants of *Synechocystis* by homologous recombination. We generated 500 mutants that extended across all the gene categories at the first level in Cyanobase (http://www.kazusa.or.jp/cyano/) (Supplementary Table 1). The coverage of ORFs is lowest in the category ‘Cell envelope’ (7.5%) and highest in the category ‘Energy metabolism’ (25.8%). The total coverage of this library is about 15% of the genome.

**Isolation of mutants with similar chlorophyll fluorescence induction kinetics to pmgA and sll1961 mutants**

We first grow the cyanobacteria in patches with a diameter of about 1 cm on plates under low-light (20 μmol m⁻² s⁻¹) or high-light (200 μmol m⁻² s⁻¹) conditions, and then the plates were dark adapted for 15 min and subjected to the fluorescence measurements. Two-dimensional fluorescence images were collected every 40 ms for 45 s starting at the onset of the orange actinic light. The intensity of the fluorescence from each patch of cyanobacteria in each image was integrated, and the resulting one-dimensional data (i.e., fluorescence kinetics) were used for the detailed analysis of the influence of the gene disruption in each mutant. Examination of the fluorescence kinetics of the sll1961 mutant [Fig. 1B (1)] and that of the pmgA mutant [Fig. 1B (2)] under high-light conditions revealed that both mutants showed a lower first peak (at around 0.5 s after the onset of the illumination) than the wild type (Fig. 1B), but the kinetics were more or less similar under low-light conditions [Fig. 1A (1, 2)], which is consistent with the findings of an earlier report (Fujimori et al. 2005). In addition, the final level of the fluorescence (at 45 s after the onset of the illumination) is higher than the initial peak in the mutants but not in the wild type only under high-light conditions. Next, we systematically determined the fluorescence kinetics of the transposon-inserted mutants and looked for the mutants that shared similar fluorescence kinetics characteristics with the sll1961 and pmgA mutants. Among 500 mutants, six mutants showed fluorescence kinetics similar to those of the sll1961 and pmgA mutants under high-light conditions [Fig. 1B (3–7)]. Under low-light conditions, however, all the mutants showed similar fluorescence kinetics to the wild type, with one exception: the slr0249 mutant showed a more pronounced difference under low-light conditions than under high-light conditions. Annotations of the genes that were disrupted in the mutants are summarized in Table 1.

**Characteristics of the photosynthetic apparatus of the isolated mutants**

Fluorescence emission spectra of the mutants and the wild-type strains were determined at 77 K in order to detect possible differences in photosystem stoichiometry between them. The fluorescence at 725 nm is predominantly emitted from PSI, while that at 695 nm arises mainly from PSII at low temperature. Thus, the ratio of fluorescence intensity at 725 nm and that at 695 nm determined at 77 K (F725/F695) is a good indicator of the PSI/PSII ratio. Fig. 2A shows the relative fluorescence intensity of the cells grown in liquid culture under low-light or high-light conditions. Cells of *ctaEI*, *ctaCI*, *ccmK2*, *slr1916* and *slr0645* mutant strains grown under low light exhibited no significant difference in the PSI/PSII ratio compared with wild-type cells.
Under high-light conditions, however, they showed a higher level of PSI/PSII compared with the wild-type cells. These characteristics are quite similar to those observed for \( \text{pmgA} \) and \( \text{sll1961} \) mutants. The results indicate that the photosystem stoichiometry of the five mutants is different from that of the wild type only under high-light conditions. In the case of the \( \text{slr0249} \) mutant, the PSI/PSII ratio was lower than that of the wild type under low-light conditions and similar to that of the wild type under high-light conditions.

The increase of \( F_{725}/F_{695} \) could be caused either by the increased level of PSI or by the decreased level of PSII. It is expected that a higher PSI level would result in a higher chlorophyll content, since nearly 90% of the chlorophyll molecules are associated with PSI in cyanobacteria. The chlorophyll content would not be affected much by the change in the level of PSII, in which the main antenna pigments are not chlorophyll but phycobiliproteins. Absorption spectra of whole cells of the wild type and the mutants suspended in BG-11 medium were measured to estimate chlorophyll contents on a cell basis. Four mutants (\( \text{sll1961}, \text{pmgA}, \text{ccmK2} \) and \( \text{slr1916} \)) showed a higher chlorophyll level than the wild type in the cells grown under high light and possibly in the cells grown under low light (Fig. 2B). On the other hand, three mutants (\( \text{ctaEI}, \text{ctaCI} \) and \( \text{slr0645} \)) did not show the increased level of chlorophyll content compared with the wild type in the cells grown under both high-light and low-light conditions. These results suggest that the cause of the high \( F_{725}/F_{695} \) ratio in the high-light grown cells of \( \text{sll1961}, \text{pmgA}, \text{ccmK2} \) and \( \text{slr1916} \) mutants is an increase in PSI content compared with the wild type. On the other hand, \( \text{slr0645} \) mutants grown under high-light conditions would have a decreased level of PSII. In the case of the \( \text{slr0249} \) mutant, a decrease of \( F_{725}/F_{695} \) was observed compared with the wild type under low-light conditions and a small decrease of chlorophyll compared with the wild type under high-light conditions. Since the growth of this mutant is rather slow under both high-light and low-light conditions (see below), it is difficult to discuss the cause of the change in photosystem stoichiometry under the possible secondary influence of the slow growth.

The six mutants were thus divided into three groups according to two parameters, \( F_{725}/F_{695} \) and the level of chlorophyll. Group I, consisting of \( \text{sll1961}, \text{pmgA}, \text{ccmK2} \) and \( \text{slr1916} \) mutants, showed a high value of both parameters under high-light conditions (Fig. 2D, solid circle) but the phenotype was weaker under low-light conditions (Fig. 2C). Group II, consisting of \( \text{ctaEI}, \text{ctaCI} \) and \( \text{slr0645} \) mutants, showed a high value of \( F_{725}/F_{695} \) but a normal value of chlorophyll content under high-light conditions (Fig. 2D, broken circle). The phenotype of the group II mutants disappeared almost completely under

**Fig. 1** Chlorophyll fluorescence kinetics of the wild type (thin line) and the mutants (bold line) under low- (A) and high- (B) light conditions. Bold lines in (1), (2), (3), (4), (5), (6), (7) and (8) indicate the \( \text{sll1961}, \text{pmgA}, \text{ccmK2}, \text{ctaEI}, \text{slr1916}, \text{ctaCI}, \text{slr0645} \) and \( \text{slr0249} \) mutants, respectively. The cells on BG-11 media were illuminated with light at 200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for 45 s to monitor fluorescence kinetics after 15 min dark adaptation. The intensity of the fluorescence was normalized with the initial value at the start of actinic light.
low-light conditions (Fig. 2C). Group III consists of only the *slr0249* mutant (Fig. 2D, square). Group III showed a lower value of $F_{725}/F_{695}$ under low-light conditions and a lower value of chlorophyll concentration under high-light conditions compared with the wild type (Fig. 2C, D).

**Growth under photomixotrophic conditions**

It was reported that growth of the *pmgA* mutant is severely suppressed under photomixotrophic conditions with 5 mM glucose and medium light at $50 \mu$mol m$^{-2}$ s$^{-1}$ (Hihara and Ikeuchi, 1997). All the mutants and the wild type could grow on the BG-11 agar plate containing 5 mM mannitol, a non-permeable solute, under a photon flux density of $100 \mu$mol m$^{-2}$ s$^{-1}$ (Fig. 3, lower part). In the presence of 5 mM glucose, however, the growth of all of the group I mutants (*pmgA*, *slr1961*, *ccmK2* and *slr1916*) was severely suppressed, while that of group II mutants (*ctaEI*, *ctaCI*, *slr0645*) was the same as that of the wild type (Fig. 3, upper part). The growth of the *slr0249* mutant was always slower than that of the wild type irrespective of the growth conditions.

**Discussion**

**Fluorescence kinetics as a tool to analyze the phenotype of mutants**

Here we demonstrate that the simple monitoring of chlorophyll fluorescence could be used to screen mutants with a defect in the regulation of photosystem stoichiometry. Out of six candidates that showed similar fluorescence kinetics to those of two known mutants (*sll1961* and *pmgA*), five mutants (*ccmK2*, *slr1916*, *ctaEI*, *ctaCI* and *slr0645*) showed a defect in the regulation of photosystem stoichiometry under high-light conditions. The remaining mutant (*slr0249*) showed the abnormal photosystem stoichiometry only under low-light conditions, which may be due to the slow growth of this mutant. In any event, the characteristic change of fluorescence kinetics in these mutants is an efficient and convenient way to find the regulatory component of photosystem stoichiometry.

It should be noted that the method presented here does not assume any theoretical relationship between fluorescence kinetics and the physiological consequences of the modified photosystem stoichiometry. Since the discovery of the ‘Kautsky effect’ of chlorophyll fluorescence, the complex kinetics of the fluorescence have attracted the interest of many people, and interpretation of the ‘meaning’ of the kinetics was always the point at issue (Govindjee 1995, Lazar 1999). In this study, however, the similarity of the ‘shape’ of the fluorescence kinetics is the only criterion to screen the mutants. Thus, there is a possibility that this method could be applied to the screening of any other regulatory components of photosynthesis. Moreover, it may even be possible to apply to the screening of factors that are not directly related to photosynthesis when we use cyanobacteria as the experimental material. In plant cells, photosynthesis is conducted in chloroplasts and, consequently, chlorophyll fluorescence principally reflects the condition of photosynthesis. In cyanobacteria, however, photosynthetic and other metabolic pathways are not separated in organelles. The respiratory electron transport chain shares several components with the photosynthetic electron transport chain, and carbohydrate metabolism and nitrogen metabolism have a relationship with photosynthesis through the production and consumption of ATP or reducing power (Scherer et al. 1988, Flores et al. 2005,

<table>
<thead>
<tr>
<th>ORF ID</th>
<th>Gene name</th>
<th>Annotation in Cyanobase</th>
<th>Category</th>
<th>PSI/PSII</th>
<th>Glucose sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>sll1961</td>
<td><em>pmgA</em></td>
<td>Photomixotrophic growth-related protein</td>
<td>Other categories</td>
<td>High PSI (HL)</td>
<td>Yes</td>
</tr>
<tr>
<td>sll1968</td>
<td><em>ccmK2</em></td>
<td>CO₂-concentrating mechanism protein CcmK</td>
<td>Photosynthesis and respiration</td>
<td>High PSI (HL)</td>
<td>Yes</td>
</tr>
<tr>
<td>slr1916</td>
<td><em>ctaEI</em></td>
<td>Cytochrome c oxidase subunit III</td>
<td>Photosynthesis and respiration</td>
<td>Low PSII (HL)</td>
<td>No</td>
</tr>
<tr>
<td>slr1136</td>
<td><em>ctaCI</em></td>
<td>Cytochrome c oxidase subunit II</td>
<td>Photosynthesis and respiration</td>
<td>Low PSII (HL)</td>
<td>No</td>
</tr>
<tr>
<td>slr0645</td>
<td>Hypothetical protein</td>
<td>Hypothetical</td>
<td>Hypothetical</td>
<td>Low PSII (HL)</td>
<td>No</td>
</tr>
<tr>
<td>slr0249</td>
<td>Hypothetical protein</td>
<td>Hypothetical</td>
<td>Hypothetical</td>
<td>High PSII (LL)</td>
<td>No</td>
</tr>
</tbody>
</table>
It has been established that all the metabolic pathways are related to each other on a metabolic network, and local perturbations in metabolite concentrations could reach the whole network (Barabasi and Oltvia 2004). Chlorophyll fluorescence from cyanobacteria would reflect the condition of not only photosynthesis but also a wide variety of metabolic systems and cellular processes, because photosynthesis in cyanobacteria is a part of metabolic networks in the same compartment. Thus, the method developed in the present study has the possibility to be used to investigate various networks other than photosynthesis. The fluorescence kinetics of the mutants could be easily registered in a database, since the data themselves are a simple one-dimensional array of rational numbers. One can search for the fluorescence kinetics in the database based on the similarity to the kinetics observed in the mutant of interest. In such a way, candidates for the factors that are involved in the process of interest could be easily listed. Such a possibility should be examined in the near future.

In this study, 500 mutants of *Synechocystis* PCC 6803 were created. Since cyanobacteria usually have about 10 copies of genomes in a cell, it is necessary to use a process to promote segregation of the genome to obtain fully segregated mutants. Our procedure to promote segregation (re-streaking of cells five times on plates containing antibiotics) does not necessarily guarantee the full segregation of the genomes. There are losses and gains in this regard. The phenotypes of the mutants may not be stable because of the possible change in the levels of segregation. On the other hand, our procedure enables the collection of information on the mutants of essential genes...
and slr1916 ometry were found. Among them, two mutants (for the regulatory component of photosystem stoichiometry extensively studied. In this study, six other candidates the regulation of photosystem stoichiometry have been reported so far, although the physiological aspects of stoichiometry under high-light conditions have been comprehensively. 

**The factors involved in the adjustment of photosystem stoichiometry**

The adjustment of photosystem stoichiometry as a response to the changes in light environments has been widely observed in cyanobacteria (Kawamura et al. 1979, Fujita et al. 1985, Manodori and Melis 1986, Hihara et al. 1998), algae (Melis et al. 1996) and higher plants (Chow et al. 1990). The dynamic alteration in the composition of thylakoid membranes 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). It is also reported that the stoichiometry is modulated by nutrient availability or affected by mutations of some genes (Melis et al. 1985). Evidence from characterization of the mutants defective in the regulation of the photosystem stoichiometry suggested that the adjustment of photosystem stoichiometry in cyanobacteria is an essential response to long-term high-light conditions (Hihara et al. 1998, Fujimori et al. 2005). Only two regulatory mutants of photosystem stoichiometry under high-light conditions have been reported so far, although the physiological aspects of the regulation of photosystem stoichiometry have been extensively studied. In this study, six other candidates for the regulatory component of photosystem stoichiometry were found. Among them, two mutants (ccmK2 and slr1916 mutant), together with the sll1961 and pmgA mutants, were clearly categorized in one group (group I) by two criteria: (i) increased PSI content under high-light conditions; and (ii) impairment in growth under photomixotrophic conditions. ccmK2 gene products are the shell proteins in carboxysomes (Cannon et al. 2002). At first glance, the inability to regulate photosystem stoichiometry does not seem to be directly related to the disruption of the ccmK2 gene. However, we could exclude the possibility of secondary mutation on sll1961 and pmgA genes in ccmK2 and slr1916 mutants by sequencing of coding regions of those genes in the mutants (data not shown). One explanation for our finding is the indirect effect of the carbon-concentrating mechanisms on the photosystem stoichiometry through the change in cellular CO2 concentration. A lowered PSI/PSII reaction center ratio was observed in Anacystis nidulans cells under low CO2 conditions, and this relative increase of PSI in cells grown in low CO2 was explained as meeting the demand to generate extra ATP through cyclic electron transfer, which is necessary to import inorganic carbon (Manodori and Melis 1984). High-light conditions increase the demand for inorganic carbon, and the ccmK2 mutant would have less activity for inorganic carbon uptake than the wild type. The increased amount of PSI in the ccmK2 mutant grown under high-light conditions might be a result of increased demand for CO2. slr1916 has an esterase domain but its function is unknown. Both mutants may affect photosystem stoichiometry through the change in photosynthetic or other metabolic pathways. It was reported that the pmgA mutant could not grow under photomixotrophic growth conditions, although the mechanism of photomixotrophic sensitivity in the mutants is totally unknown (Hihara and Ikeuchi, 1997). It is now clear that the growth of the mutants of group I (pmgA, slr1961, ccmK2 and slr1916) was severely suppressed under photomixotrophic conditions (Fig. 3). A defect in the decrease of PSI content under high-light conditions must be related to the growth sensitivity under photomixotrophic conditions, but the mechanism of this sensitivity is currently unknown.

**Group II mutants** seem to have a lower PSII content under high-light conditions. Among them, we found two mutants that have a defect in cytochrome c oxidase. Cytochrome c oxidase, composed of the three subunits CtaC, CtaD and CtaE, is a component of the respiratory chain that catalyzes the reduction of oxygen to water and generates an electrochemical potential that can provide energy for numerous cellular processes. It was reported that over-reduction of the plastoquinone pool presumably takes places in thylakoid membranes of a cytochrome c oxidase mutant (Kufryk and Vermaas 2006). In addition, cytochrome c oxidase mutants were reported to exhibit a decreased level of PSII with little loss of chlorophyll content (Nomura et al. 2006). A close look at the chlorophyll fluorescence kinetics in the ctaEI and ctaCI mutants (Fig. 1) revealed a transient decrease of chlorophyll fluorescence...
intensity at 40 and 80 ms after onset of the actinic light. The decrease seemed to be explained by the oxidation of the plastoquinone pool that had been reduced in the dark in the absence of cytochrome c oxidase. Cytochrome c oxidase is the major terminal acceptor for electrons from the plastoquinone pool in the dark, while PSI should be the primary electron acceptor from the plastoquinone pool in the light. The observed change in the photosystem stoichiometry could be the result of a reduced plastoquinone pool in the mutants. Another mutant in group II is that of slr0645. slr0645 encodes a hypothetical protein with a von Willebrand factor type A (vWA) domain. Since this domain is supposed to serve for protein–protein interaction, regulation of photosystem stoichiometry through such an interaction with other component(s) may be possible.

In this study, 15% of the ORFs in the genome were screened, and at least five factors involved in the regulation of photosystem stoichiometry through such an interaction with other component(s) may be possible.

In this study, 15% of the ORFs in the genome were subjected to the analysis. We suggest that >30 factors would be identified if all the ORFs of the genome were subjected to the analysis. We are currently expanding our system to clarify the whole image of the regulation of photosystem stoichiometry.

Materials and Methods

In vitro transposon mutagenesis and sequence analysis

Transposon mutagenesis on cosmid clones containing the \textit{Synechocystis} sp. PCC 6803 genome fragment (Kaneko et al. 1996) was carried out using the pGPS-1 plasmid and the GPS-1 genomepriming system (New England Biolabs, Beverly, MA, USA) according to the manufacturer's protocol. Mutagenized cosmid DNA was transferred into \textit{E. coli} and transformants were plated onto selective media containing ampicillin (50 \textmu g mg\textsuperscript{-1}) and chloramphenicol (20 \textmu g mg\textsuperscript{-1}). The mutated cosmids were recovered from the transformed \textit{E. coli} cells, and the site of the transposon insertion was identified by direct sequencing with the primer 5'-CACAGCATAACTGGACTGATTTCCAG.

Strains and growth conditions

The glucose-tolerant wild-type strain \textit{Synechocystis} sp. PCC 6803 was grown in BG-11 medium (Rippka et al. 1979) buffered with 10 mM \textit{N}-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH (pH 8). Cells were grown under continuous illumination at 30\degree C. Liquid cultures were bubbled with air. 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. A photon flux density at 20, 100 and 200 \textmu mol photons m\textsuperscript{-2}s\textsuperscript{-1} was regarded as low, medium and high light, respectively. For the creation of site-directed mutants, the wild-type strain was transformed by the mutated cosmid described above, and chloramphenicol-resistant clones were selected on solid media. Resistant colonies were serially re-streaked at least five times on BG-11 agar plates supplemented with antibiotics (8.0 \textmu g of chloramphenicol ml\textsuperscript{-1}) to promote the segregation of the mutant genome. The \textit{pmgA} \textit{slr1961} mutant was created by insertion of the spectinomycin-resistant cassette (Hihara and Ikeuchi 1997). The \textit{slr1961} mutant was generated by the insertion of a transposon that has a chloramphenicol-resistant cassette (Fujimori et al. 2005). Site-directed mutants and the \textit{slr1961} mutant were maintained with 8 \mu g ml\textsuperscript{-1} chloramphenicol, while the \textit{pmgA} mutant was maintained with 20 \mu g ml\textsuperscript{-1} spectinomycin.

Monitoring of chlorophyll fluorescence kinetics

A 10\mu l aliquot of cell culture, of which the OD\textsubscript{730} was adjusted to 0.5 by BG-11 liquid medium, was dropped on BG-11 agar plates. The wild-type cells gave 1.1 \times 10\textsuperscript{5} cells ml\textsuperscript{-1} at 1 OD\textsubscript{730}, when the OD was determined by a spectrophotometer (GeneSpec III, Hitachi, Tokyo, Japan). After incubation at 30\degree C under light at 20 \mu mol m\textsuperscript{-2}s\textsuperscript{-1} for 72 h or at 200 \mu mol m\textsuperscript{-2}s\textsuperscript{-1} for 48 h, the plates were subjected to the measurement of chlorophyll fluorescence kinetics. Cells on agar plates were dark adapted for 15 min before the measurements. The plates were put in a fluorescence imaging system (FluorCam 700MF, Photon System Instruments, Brno, Czech Republic) and actinic light (200 \mu mol m\textsuperscript{-2}s\textsuperscript{-1}) was applied for 45 s to monitor chlorophyll fluorescence. The actinic light was generated by two panels of orange light-emitting diodes (HLMP EH08, Agilent Technologies, Wilmington, MA, USA) with a peak wavelength of 615 nm, and the spectral half-width is 18 nm. A fluorescence image was captured every 0.04 s for 45 s by a CCD camera (ICX429AL, Sony, Tokyo, Japan) that produced 752 \times 580 pixel images in a 12-bit gray scale. The sensitivity was adjusted to 20\% on the FluorCam software. The fluorescence at around 700 nm was detected by filtering the emission through a custom-made interference filter with a peak at 700 nm (the full-width at half-maximum is 30 nm) and a red blocking filter (RG697, Corion, Franklin, MA, USA). The fluorescence intensity was normalized with the initial value at the start of actinic light.

Absorption and 77 K fluorescence emission spectra

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 placed just in front of the photomultiplier. The concentration of chlorophyll was calculated by the equations of Arnon et al. (1974). The chlorophyll content of cells was obtained by normalization at OD\textsubscript{730}, which was determined by a spectrophotometer (Model 356; Hitachi, Tokyo, Japan). Low temperature fluorescence emission spectra at 77 K were recorded using a custom-made apparatus (Sonoike and Terashima 1994). Cells containing 5 \mu g chlorophyll ml\textsuperscript{-1} in BG-11 medium were placed in a sample holder and excited by blue light passing through a broad band-pass filter (CS 4-96, Corning Inc., NY, USA). Before measurement, cells were incubated in darkness for 10 min at room temperature to eliminate the possible effects of state transition.

Supplementary material

Supplementary material mentioned in the article is available online to subscribers at the journal website www.pcp.oxfordjournals.org.

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References


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