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Targeted Mutagenesis of ORF326, *frxC* and ORF469 of a Cyanobacterium, *Synechocystis* PCC6803, Homologous to Liverwort Chloroplast Genes

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ORF326, *frxC* and ORF469 of a transformable cyanobacterium, *Synechocystis* PCC6803, have sequence similarity with ORF316, *frxC* and ORF465 on the chloroplast genome of a liverwort, *Marchantia polymorpha*, respectively. To elucidate their functions, targeted mutagenesis was performed by transformation with cloned DNA in which the ORF was disrupted by insertion of a kanamycin resistance gene cassette. Streak-purifications of a single colony of each transformant were repeated to segregate homozygous mutants for disrupted copies, because *Synechocystis* PCC6803 was reported to have approximately 10 chromosomal DNA copies. Southern blot analysis revealed that mutants for ORF326 had not only disrupted ORF326 copies but also wild type ORF326 copies. This suggests that ORF326 is indispensable for growth under the mixotrophic growth condition used. However, mutants for *frxC* and mutants for ORF469 had only mutated copies, indicating that they are dispensable for growth. Growth and chlorophyll *a* content of an ORF469-disrupted mutant were compared to those of the wild type under mixotrophic growth condition, but no significant difference was detected. This indicates that ORF469 is required for neither normal growth nor chlorophyll biosynthesis under this condition.

Key words: Cyanobacteria, Gene disruption, *Synechocystis* PCC6803

INTRODUCTION

Cyanobacteria carry out plant-like oxygenic photosynthesis highly simi-

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lar to plant chloroplasts. The unicellular cyanobacterium *Synechocystis* PCC6803 is very amenable to molecular genetic analysis. It is naturally competent, transformable by exogenous DNA (Grigorieva and Shestakov 1982) and can be grown mixotrophically on glucose (Rippka *et al.* 1979), and these features enabled targeted mutagenesis of PSII genes (Williams 1988). Therefore we used *Synechocystis* PCC6803 as a model system to elucidate functions of open reading frames (ORFs), ORF316, *frxC* and ORF465, on the chloroplast genome of a liverwort, *Marchantia polymorpha* (Ohyama *et al.* 1988). ORF316 encodes a polypeptide with one zinc-finger motif, found in DNA-binding proteins (Evans and Hollenberg 1988). On the liverwort chloroplast genome *frxC* and ORF465 form gene cluster and encode polypeptides that have sequence similarity to iron protein and β subunit of molybdenum-iron protein of nitrogenase complex, respectively (Mevarech *et al.* 1980, Wang *et al.* 1988). However, the counterpart of *frxC* or ORF465 has not been deduced from the complete chloroplast DNA sequence of tobacco (Shinozaki *et al.* 1986) or rice (Hiratsuka *et al.* 1989). Previously, we reported cloning and nucleotide sequence of *Synechocystis* ORF326, *frxC* and ORF469, homologous to liverwort ORF316, *frxC* and ORF465, respectively (Ogura *et al.* 1991, 1992). Here we report targeted disruption of ORF326, *frxC* and ORF469 by insertional inactivation with a kanamycin resistance gene cassette (Fig. 1) and primary characterization of an ORF469-disrupted mutant.

MATERIALS AND METHODS

1. *Culture conditions*

Culture conditions of transformants and wild type *Synechocystis* PCC6803 were described previously (Ogura *et al.* 1991), except where noted otherwise.

2. *Plasmid construction*

DNA fragment containing the ORF to be disrupted was subcloned into a plasmid vector, and a kanamycin resistance gene cassette from pUC4K (Oka *et al.* 1981, Vieira and Messing 1982) was inserted at the site in the coding region of the ORF in the same direction. For ORF326, the 5.1 kb *Kpn*I-*Hin*dIII fragment from pSH64-1 (Ogura *et al.* 1991), was subcloned into pUC18 (Yanisch-Perron *et al.* 1985) and the 1.3 kb *Pst*I fragment from pUC4K was inserted at a unique *Pst*I site resulted in pSKH505. For *frxC*, pSHc201, which has the 1.7 kb *Hin*cII insert from the cloned 9.1 kb *Hin*dIII fragment (Ogura *et al.* 1992), in pBluescript II SK+(Alting-Meess and Short

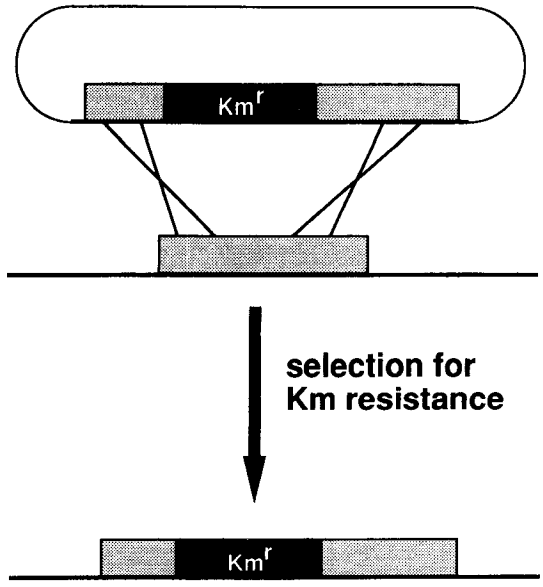

Fig. 1. Scheme of targeted mutagenesis. Thick lines represent the chromosome and cloned fragments of cyanobacterial DNA. A dotted box and a filled box show the gene to be disrupted and the kanamycin resistance gene cassette, respectively. The region from the *E. coli* vector is shown by an oval line. Each point of recombination is indicated by an X. Selection for kanamycin resistance results in replacement of wild type gene to mutated allele, which has been inactivated by insertion of the kanamycin resistance gene cassette, via homologous recombination.

1989), was digested with *Acc*I and treated with T4 DNA polymerase to convert protruding ends to blunt. Then the linearized plasmid was ligated to the 1.3 kb *Hin*cII fragment conferring kanamycin resistance resulted in pSFC201. For ORF469, the 2.3 kb *Sma*I-*Hin*dIII fragment from the 9.1 kb *Hin*dIII fragment, was subcloned into pUC13 (Messing 1983). The resultant plasmid pSSH201 was cut at a unique *Kpn*I site and treated with T4 DNA polymerase to make ends blunt. Then the linearized plasmid was ligated to the 1.3 kb *Hin*cII fragment from pUC4K resulted in pSSH202.

3. *Transformation of Synechocystis PCC6803*

Transformation of *Synechocystis* PCC6803 was performed with the plasmids described above, according to the established procedure (Williams 1988). For selection, the transformation mixture was spread directly on solid BG-11 medium (Stanier *et al.* 1971), supplemented with 1.5 % (w/v) agar, 0.3 % (w/v) sodium thiosulfate and 10 mM TES-KOH pH 8.2, containing 10 μg/ml kanamycin, 10 mM glucose and 10 μM DCMU and incubated at 26 °C under continuous illumination. Kanamycin resistant colonies were restrea-

ked on the solid medium of the same composition for several times.

4. *Southern hybridization*

The 0.9 kb *Pst*I-*Eco*RI fragment from pSH64-1, the 0.46 kb *Hae*III fragment from pSHc201 and the 0.65 kb *Pst*I-*Acc*II fragment from pSSH202 were labeled with [α-^{32}P]dCTP as described previously (Ogura *et al.* 1991) and used as probes for ORF326, *frxC* and ORF469, respectively. Southern hybridization was performed under high stringency condition, at 42 °C for 16 hr in hybridization buffer (50 % formamide, 1 × Denhardt's solution, 5 × SSC, 0.1 % SDS, 0.1 mg/ml heat denatured calf thymus DNA) with ^{32}P-labeled probes prepared as above (Sambrook *et al.* 1989).

5. *Primary characterization of transformants*

Wild type *Synechocystis* PCC6803 and ORF469-disrupted mutants were cultured as described previously, except that BG-11 medium was supplemented with 10 mM glucose. Their growth was monitored by measuring optical density (OD) at 730 nm and their chlorophyll *a* content was measured according to Tandeau de Marsac and Houmard (1988).

RESULTS AND DISCUSSION

1. *Disruption of ORF326*

For targeted disruption of ORF326, a plasmid, pSKH505, containing inactivated ORF326 by insertion of a kanamycin resistance gene cassette at the coding region, was constructed. The plasmid was used in circular form to transform *Synechocystis* PCC6803 according to Williams (1988) and selection was performed on solid BG-11 medium with 10 μg/ml kanamycin, 10 mM glucose and 10 μM DCMU, so that the PSII deficient mutant could grow because possible involvement of ORF326 in regulation of genes encoding components of PSII complex could not be excluded. Kanamycin resistant colonies obtained were restreaked on solid medium with the same composition as used for selection to segregate homozygous mutants, because *Synechocystis* PCC6803 has been reported to have approximately ten chromosomal DNA copies (Williams 1988, Labarre *et al.* 1989). After several times of restreaking, three transformants were subjected to liquid culture. DNA from each of them and wild type was digested with *Eco*RI or *Hin*dIII and used for Southern hybridization with a specific probe for ORF326. For all transformants this probe hybridized to the 3.0 kb *Eco*RI fragment and the 5.5 kb *Hin*dIII fragment corresponding to wild type ORF326 copies, in addition to the 4.2 kb *Eco*RI fragment and the 2.1 kb *Hin*dIII fragment for

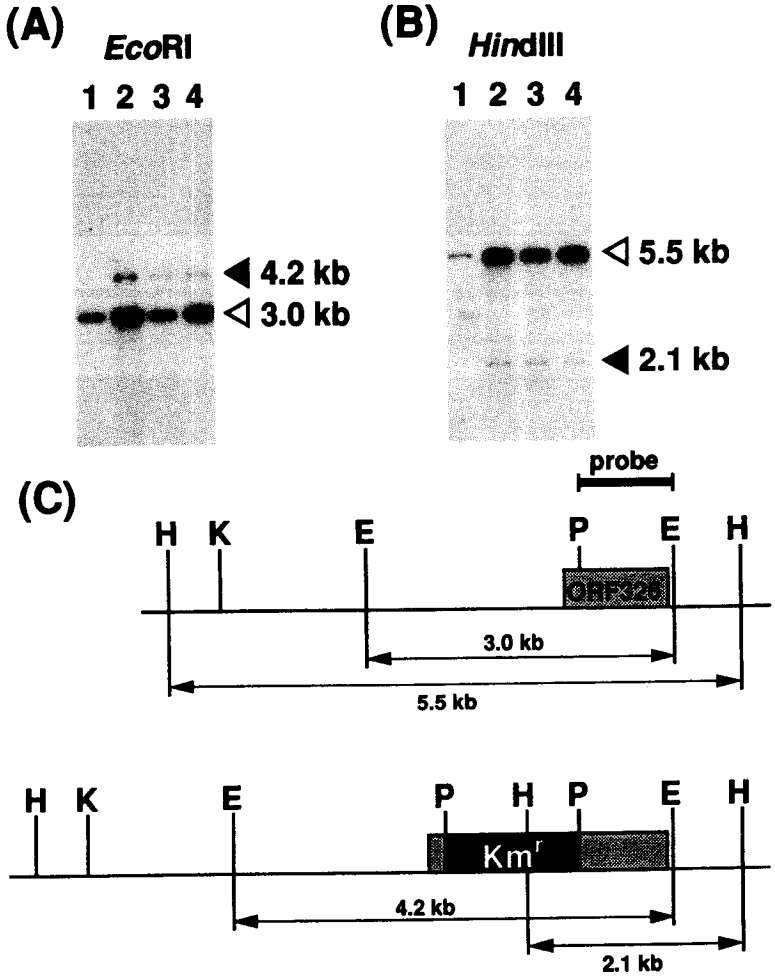

Fig. 2. Southern blot analysis of genomic DNA from transformants with disrupted ORF326. (A) and (B) DNA isolated from wild type (lane 1) and three individual transformants (lanes 2-4) was digested with *Eco*RI (A) or *Hin*dIII (B) and hybridized with ORF326-specific probe indicated in (C). The signals corresponding to wild type ORF326 and disrupted copy are indicated by open and filled arrowhead with size, respectively. (C) Restriction maps of regions containing wild type ORF326 (upper) and disrupted ORF326 (lower). Horizontal thin lines represent the chromosome of *Synechocystis* PCC6803. ORF326 and the kanamycin resistance gene cassette are indicated by the dotted and the filled boxes, respectively. The region used as a probe is indicated by a thick bar above. Vertical lines indicate restriction sites. E: *Eco*RI, H: *Hin*dIII, K: *Kpn*I and P: *Pst*I.

disrupted ORF326 copy (Fig. 2). Transformants homozygous for disrupted

ORF326 were not obtained either by further rounds of restreaking or in other rounds of transformation experiments (data not shown). These findings indicate that ORF326 is required for growth under this selection condition.

In *E. coli*, mutational analysis of *dedB*, homologous to ORF326, has been reported, but mutants containing no wild type *dedB* were not obtained, suggesting *dedB* is indispensable (Nagano *et al.* 1991). Recently, *dedB* was indicated to encode β subunit of carboxyltransferase component of acetyl-CoA carboxylase and was renamed *accD* (Li and Cronan 1992b). Acetyl-CoA carboxylase catalyzes the first committed step of fatty acid synthesis. The enzyme catalyzes two distinct half-reactions; the carboxylation of biotin with bicarbonate and following transfer of the CO_2 group from carboxy-biotin to acetyl-CoA to form maronyl-CoA, which is a substrate for fatty acids synthesis. In *E. coli*, the enzyme consists of three different components, biotin carboxylase, biotin carboxyl carrier protein (BCCP) and carboxyltransferase. Since *Synechocystis* PCC6803 is a prokaryote, ORF326 seems to be involved in fatty acid synthesis as is the case in *E. coli accD*, and this is supported by the failure to segregate transformants homozygous for mutated ORF326, described above. Harder *et al.* (1972) isolated temperature-sensitive mutants of *E. coli* defective in fatty acid synthesis. Among them, *fabE* mutant was found to have G to A transversion that converts 100th glycine, close to the attachment site of biotin, of BCCP to serine (Li and Cronan 1992a). So conditional ORF326 deficient mutants such as *fabE* mutant, will be utilized for further functional analysis.

In plants, fatty acid biosynthesis in leaves occurs in the chloroplasts where acetyl-CoA carboxylase is also localized, but all three functional domains are thought to be present on one high molecular weight subunit of molecular mass over 200 kD, like in animals (Hellyer *et al.* 1986), as shown in maize (Egli *et al.* 1993). Recently, a polypeptide that crossreacted with an antibody against the truncated product of pea *accD* (formerly called *zfpA*) expressed in *E. coli*, was detected in pea chloroplasts, and acetyl-CoA carboxylase activity in soluble extract from pea chloroplasts was inhibited by the antibody (Sasaki *et al.* 1993). The deduced product of liverwort ORF316 has sequence similarity to pea *accD* product but does not have a region corresponding to the highly acidic N-terminal region characteristic to pea *accD* product. Therefore, whether liverwort ORF316 encodes a component of acetyl-CoA carboxylase or not is to be determined, and biochemical analysis will give light to this problem.

2. *Disruption of frxC and ORF469*

For targeted disruption of each of *frxC* and ORF469, plasmids pSFC201 and pSSH202 containing inactivated copy were used, respectively. Transformation of *Synechocystis* PCC6803 was carried out with the same procedure as that of ORF326. DNA from transformants was subjected to Southern blot analysis using specific probes for *frxC* and for ORF469. As Fig. 3 shows, for all transformants obtained with pSFC201, a signal at 4.2 kb for mutated *frxC* was detected but any signal at 2.9 kb for wild type *frxC* was not. An ORF469 specific probe also hybridized to the 5.1 kb fragments for mutated copies but not to the 3.8 kb fragment for wild type copy in the *Kpn*I digests in all transformants obtained using pSSH202 (Fig. 4). Thus both transformants homozygous for mutated *frxC* and for ORF469 were obtained, and this indicates that neither *frxC* nor ORF469 is indispensable under the mixotro-

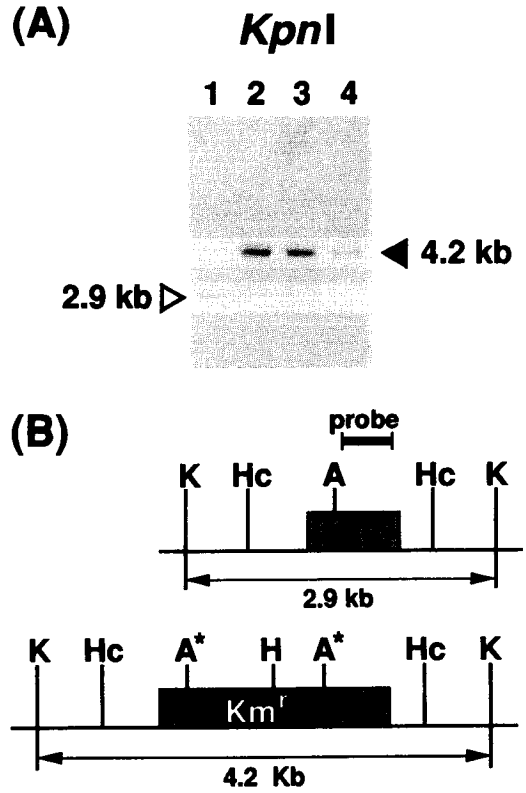

Fig. 3. Southern blot analysis of genomic DNA from transformants with disrupted *frxC*. (A) DNA isolated from wild type (lane 1) and three individual transformants (lanes 2-4) was digested with *Kpn*I and hybridized with *frxC* specific probe indicated in (B). (B) Restriction maps of regions containing wild type *frxC* (upper) and disrupted copy (lower). The dotted box indicates *frxC*. A : *Acc*I, Hc : *Hinc*II and K : *Kpn*I. Asterisks indicate inactivation of restriction sites. Others are the same as in Fig. 2. (C).

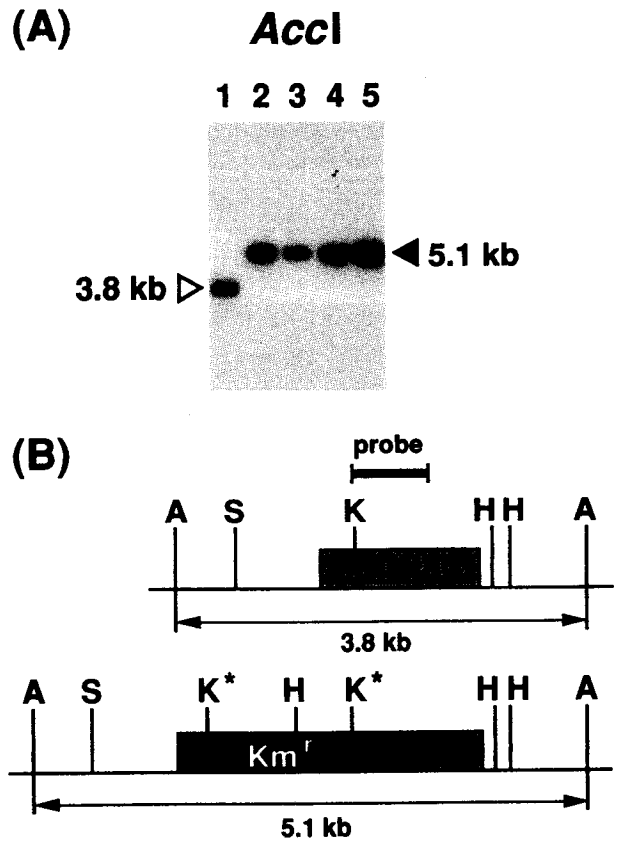

Fig. 4. Southern blot analysis of genomic DNA from transformants with disrupted ORF469. (A) DNA isolated from wild type (lane 1) and four individual transformants (lanes 2-5) was digested with *Acc*I and hybridized with ORF469 specific probe indicated in (B). (B) Restriction maps of regions containing wild type ORF469 (upper) and disrupted copy (lower). ORF469 is indicated by the dotted box. A : *Acc*I, H : *Hin*dIII, K : *Kpn*I and S : *Sma*I. Others are the same as in Fig. 3. (B).

phic selection condition. One of these ORF469-disrupted mutants was used for primary characterization and its growth and chlorophyll *a* content was compared to those of the wild type in BG-11 medium supplemented with 10 mM glucose under continuous illumination. Their growth was monitored by optical density at 730 nm, and chlorophyll *a* content was measured according to Tandeu de Marsac and Houmard (1988). As Fig. 5 shows, either growth or chlorophyll *a* content of ORF469-disrupted mutant was not significantly different from that of the wild type. This indicates that ORF469 is not required for normal growth or chlorophyll biosynthesis under a mixotrophic growth condition.

In *Chlamydomonas reinhardtii*, a chloroplast gene, *chlN* (formerly called

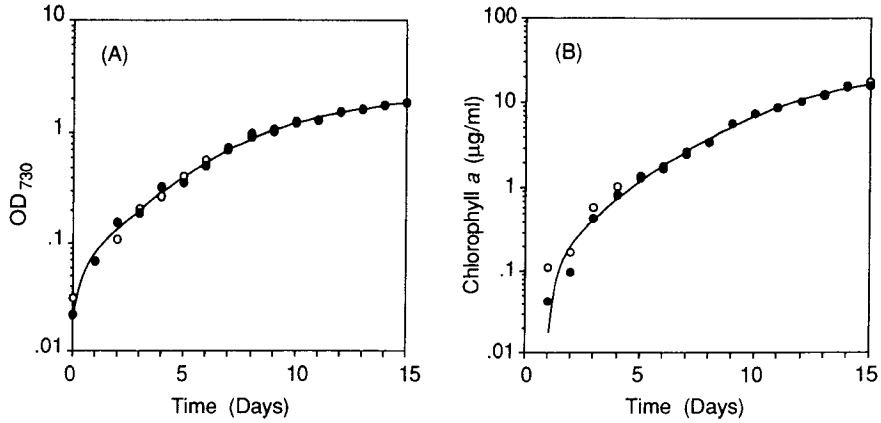

Fig. 5. Growth and chlorophyll *a* content of wild type and ORF469-disrupted mutant cells in BG-11 medium. The growth of cultures was monitored by measuring their optical density at 730 nm (A) and chlorophyll *a* content was also measured (B). Open circle and filled circle represent wild type and ORF469 deficient mutant, respectively. Data presented as a semilog plot are means of three separate cultures.

gidA), which is homologous to the liverwort ORF465, was shown to be involved in light-independent biosynthesis of chlorophyll, probably at the step of reduction of protochlorophyllide, by chloroplast transformation of H13 mutant with wild type DNA fragments (Choquet *et al.* 1992). The chloroplast mutant H13 which has chloroplast DNA deletions was yellow when grown in the dark, while the wild type is green in the dark (Goldschmidt-Clermont *et al.* 1990). However, chloroplast mutants which show a similar phenotype to H13, were green when grown in the light (Roitgrund and Mets 1990) and this is consistent with our results that the ORF469-disrupted mutant was green in the light. ORF465 homologues have been also detected in the chloroplast genomes of two conifers, lodgepole pine (*Pinus contorta*) and Norway spruce (*Picea abies*) through heterologous hybridization probed with DNA fragment containing *gidA* from chloroplast of *Chlamydomonas reinhardtii* (Lidholm and Gustafsson 1991). Furthermore, they showed, from nucleotide sequence analysis, a liverwort *frxC* homologue is located upstream of *gidA* on the chloroplast DNA of the lodgepole pine. In a filamentous cyanobacterium, *Plectonema boryanum*, a liverwort chloroplast *frxC* homologue was isolated (Fujita *et al.* 1991). Mutational analysis was performed in *Plectonema boryanum* and accumulation of protochlorophyllide was observed for transformants, homozygous for mutated *frxC* copies, when grown in dark (Fujita *et al.* 1992). A liverwort *frxC* homologue was also found in the chloroplast DNA from *Chlamydomonas reinhardtii* (Haung and Liu 1992). Suzuki and Bauer (1992) reported that mutants

homoplastic for mutated *frxC* (also termed *chlL*) copies showed the yellow-in-the-dark phenotype resulted from inability for light-independent chlorophyll synthesis. They also showed that *Chlamydomonas frxC* hybridized to the DNA from bacteria and non-flowering plants that are green in the dark, but not to the DNA from angiosperms which require light for chlorophyll synthesis, suggesting a possible correlation between the presence of *frxC* homologues and capability to synthesize chlorophyll in the dark.

Synechocystis PCC6803 can grow under dim light, but not in the dark, even if supplemented with glucose. Recently, a heterotrophic condition, termed light activated heterotrophic growth (LAHG) condition, complete darkness except for 5 min of light every 24 hr supplemented with glucose, was explored (Anderson and McIntosh 1991). In LAHG, blue light, 400-500 nm is required possibly as a signal regulating metabolic pathway but does not serve as a source of metabolic energy via photosynthetic electron transport process, for either *psbA* mutant or *psaA* mutant, which cannot grow under continuous light with glucose, grew under an LAHG condition (Anderson and McIntosh 1991, Smart *et al.* 1991). When wild type *Synechocystis* PCC6803 was grown under an LAHG condition, the amount of chlorophyll per cell dropped more than 4-fold compared to that under mixotrophic condition, and it was thought to be primarily due to a reduction in the amount of PSI complex (Smart *et al.* 1991). A similar decrease in chlorophyll per cell was observed in *Plectonema boryanum* grown in the dark (Fujita *et al.* 1992). Therefore, in *Synechocystis* PCC6803 grown under LAHG conditions, chlorophyll seems to be synthesized via a light-independent pathway, but whether a light-dependent pathway also contributes to chlorophyll synthesis under an LAHG condition or not is to be determined.

As described above, *frxC* homologues and ORF469 homologues seem to be involved in the reduction of protochlorophyllide in other organisms. In a unicellular cyanobacterium, *Synechococcus* PCC6301, the activity of light independent protochlorophyllide oxidoreductase was detected in a plasma membrane preparation (Peschek *et al.* 1989). A similar isolation and assay procedure might be applicable for *Synechocystis* PCC6803 and such biochemical analysis will help to elucidate the functions of *frxC* and ORF469 products in more detail. The mutants deficient for *frxC* or ORF469, obtained in this study, will be useful for such biochemical analysis, and they can be used to construct mutants, in which a specific amino acid, such as conserved cysteine residue, in *frxC* or ORF469 product, is substituted by transformation with DNA carrying modified copies, as is the case in the functional analysis of *psbE* and *psbF* (Pakrasi *et al.* 1991).

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ゼニゴケ葉緑体遺伝子と相同性を持つラン藻 *Synechocystis* PCC6803 株の ORF326, *frxC* および ORF469 を標的にした変異の導入

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ゼニゴケ葉緑体 ORF316, *frxC* および ORF465 と相同性を持つ，形質転換型ラン藻 *Synechocystis* PCC6803 株の ORF326, *frxC* および ORF469 の機能についての情報を得るため，これらの欠損株の作製を行った。コード領域にカナマイシン耐性遺伝子カセットを挿入することにより不活性化された変異型の ORF（オープンリーディングフレーム）を持つプラスミドを用いて *Synechocystis* PCC6803 株の形質転換を行い，カナマイシンを含む培地で選抜した。*Synechocystis* PCC6803 株は約10コピーのクロモソームを持つが，サザンブロット解析の結果，ORF326 については，変異型と野生型 ORF326 の双方を持つ株しか得られず，増殖に必要と推測された。一方 *frxC* および ORF469については，ともに全て変異型に置き換わった株が得られ，増殖には必要ないことが示された。さらに ORF469 欠損株を光照射下で培養し，増殖速度とクロロフィル *a* 濃度を測定したが，いずれも野生株とほぼ同じであり，ORF469 は，この条件下では，増殖やクロロフィル生合成に必要ないと推察された。

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