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Inactivation of a *Synechocystis* sp Strain PCC 6803 Gene with Homology to Conserved Chloroplast Open Reading Frame 184 Increases the Photosystem II-to-Photosystem I Ratio

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A gene of the unicellular cyanobacterium *Synechocystis* sp strain PCC 6803 that is homologous to the conserved chloroplast open reading frame *orf184* has been cloned and sequenced. The nucleotide sequence of the gene predicts a protein of 184 amino acids with a calculated molecular mass of 21.5 kD and two membrane-spanning regions. Amino acid sequence analysis showed 46 to 37% homology of the cyanobacterial *orf184* with tobacco *orf184*, rice *orf185*, liverwort *orf184*, and *Euglena gracilis orf206* sequences. Two *orf184*-specific mutants of *Synechocystis* sp PCC 6803 were constructed by insertion mutagenesis. Cells of mutants showed growth characteristics similar to those of the wild type. Their pigment composition was distinctly different from the wild type, as indicated by an increase in the phycocyanin-to-chlorophyll ratio. In addition, mutants also had a two- to threefold increase in photosynthetic electron transfer rates as well as in photosystem II-to-photosystem I ratio—a phenomenon hitherto not reported for mutants with altered photosynthetic characteristics. The observed alterations in the *orf184*-specific mutants provide strong evidence for a functional role of the *orf184* gene product in photosynthetic processes.

INTRODUCTION

Considerable sequence data on plastid genomes have been accumulated during recent years. The plastid DNA from several sources has been completely sequenced, including liverwort (Ohyama et al., 1986), tobacco (Shinozaki et al., 1986), rice (Hiratsuka et al., 1989), *Epifagus virginiana* (Wolfe et al., 1992a, 1992b), and *Euglena gracilis* (Hallick et al., 1993). In addition to genes encoding rRNAs, tRNAs, and proteins that are mostly involved in housekeeping and photosynthesis, all plastid genomes have been found to contain several open reading frames (ORFs). Ten of these ORFs found in tobacco chloroplast DNA are also present in the genomes of liverwort and rice (Shimada and Sugiura, 1991). *Euglena gracilis* (Hallick et al., 1993) and *Epifagus virginiana* (Wolfe et al., 1992a, 1992b) plastid DNAs each contain three of these conserved ORFs. The retained primary structure of the ORFs even from different phylogenetic groups indicates a functional role for these putative genes.

Possible functions for some of these ORFs have been predicted on the basis of homology with already known genes (Maurizi et al., 1990; Li and Cronan, 1992; Wolfe, 1994). An alternative approach to gain new information about putative ORF proteins would be to examine the phenotypic consequences of their inactivation. Although it is feasible to target gene interruptions or deletions to the chloroplast genome of plants (Svab et al., 1990; Staub and Maliga, 1992), such experiments can be performed more quickly and easily with the prokaryotic cyanobacteria. Most cyanobacterial and chloroplast genes show a remarkable degree of sequence homology, and in some cases, the same gene organization has also been reported (Steinmüller et al., 1989; Anderson and McIntosh, 1991). Thus, cyanobacteria, which according to the endosymbiont hypothesis (Gray, 1991) are the ancestors of chloroplasts, can be expected to contain genes homologous to most chloroplast ORFs. The isolation of several chloroplast ORF sequences from cyanobacteria with the aid of heterologous probes has been reported (Steinmüller et al., 1989; Ogura et al., 1991; Vörös et al., 1993), suggesting considerably high homology between conserved chloroplast and corresponding cyanobacterial ORFs.

The cyanobacterial strain *Synechocystis* sp strain PCC 6803 used in this study is naturally transformable by exogenous DNA (Grigorieva and Shestakov, 1982) and can grow (photo)heterotrophically without photosynthetic activity (Rippka, 1972; Williams, 1988; Anderson and McIntosh, 1991). Using gene replacement via homologous recombination, many directed mutations have been introduced into photosynthetic genes,

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altering the structure and function of photosystem II (PSII; reviewed in Pakrasi and Vermaas, 1992) or photosystem I (PSI) reaction centers, respectively (Chitnis et al., 1991, 1993; Smart et al., 1991, 1994).

In this study, we report the cloning and sequencing of a *Synechocystis* sp PCC 6803 gene that has significant homology with chloroplast *orf184* (*orf185* and *orf206*), which is conserved in all completely sequenced chloroplast genomes, with the exception of *Epifagus* plastid DNA (Wolfe et al., 1992b). To determine the function of the ORF184 protein, we constructed *orf184* mutants of *Synechocystis* sp PCC 6803 by insertional inactivation and deletion. Initial characterization of these mutants is reported, and the effect of *orf184* inactivation on photosynthesis in *Synechocystis* 6803 cells is discussed.

RESULTS

Molecular Cloning and Sequence Analysis

Screening of a λ genomic library of *Synechocystis* sp PCC 6803 with a 420-bp EcoRV probe from tobacco, bearing part of the chloroplast *orf184* sequence, revealed positive clones found to contain homologous fragments of cyanobacterial DNA. A part of an ORF with homology to the chloroplast *orf184* was initially identified on a 1.4-kb EcoRI-HindIII fragment (data not shown). Further digestion of positive phage clones with restriction endonucleases and probing of DNA gel blots demonstrated that the entire ORF is located on a 3.2-kb HindIII fragment of *Synechocystis* sp PCC 6803 DNA. The 3.2-kb HindIII fragment (Figure 1) was subcloned into the pBluescript KS+ vector. The nucleotide sequence of the region containing *orf184* has been completely determined on both strands. Figure 2 shows a 997-bp portion of the nucleotide sequence and presents the deduced amino acid sequence of the potentially encoded ORF184 protein. A putative ribosome binding site (5′-GAGAAA-3′) was

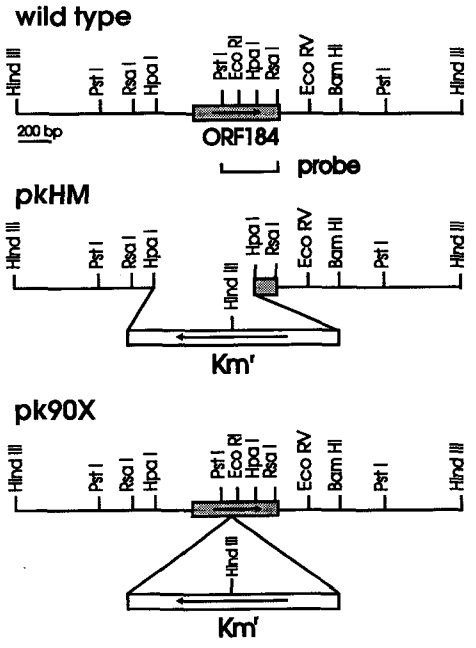

Figure 1. Scheme for Inactivation of *orf184* in *Synechocystis* sp PCC 6803.

A restriction map of the genomic region of *Synechocystis orf184* and a strategy for constructing the donor plasmids with inactivated (pk90X) and deleted (pkHM) *orf184* copies are shown. Arrows within the boxes representing the genes indicate the direction of transcription. Km′, kanamycin resistance cartridge (open boxes); shaded boxes represent *orf184* sequences.

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GTTAACCCAGGGGGGTTGGGGAGATGAAAAAAGTTATTGCCTTCGTATTCC      51
TCCTTCTGGGACCGTTTGAAAAGTCCCCTAGCTCCCAAAATTTGGTAGAGG     102
TTAGACCCCAGTTCCCCTAAGGTGATCGCCCTATCATCCTAGCCCCTAGCC     153
                                                    RBS
CTGATGGGGTAAAATTCAATGGTTAGTTTCGTTGTAATTATTGTTAGAGAA     204
ATACATGGGTGGACAGACGCTCGCAGAATCTTCCCAAGTTTTGCGCCAAGA     255
    M  G  G  Q  T  L  A  E  S  S  Q  V  L  R  Q  E
AGTATTGGGAGCCCGCCGCTTCAGCAACTTTTTCTGGGCTGGAATTTCTAC     306
  V  L  G  A  R  R  F  S  N  F  F  W  A  G  I  S  T
CATTGGTGGTGTCGGTTTCCTGTTGGCCGGCCTTTCTAGCTACTTTGGCAA     357
  I  G  G  V  G  F  L  L  A  G  L  S  S  Y  F  G  K
AAATCTTTTGATTGTTAGTGACACCACCGGACTGCAGTTTATTCCCCAGGG     408
  N  L  L  I  V  S  D  T  T  G  L  Q  F  I  P  Q  G
TGTGGCGCTACTTTTCTACGGTGTGGCCGGCAGCACAGTGGCAGGCTATCT     459
  V  A  L  L  F  Y  G  V  A  G  S  T  V  A  G  Y  L
CTGGCTAACCATGGCTCTAAACGTCGGCAGTGGTTACAACGAATTCAATAA     510
  W  L  T  M  A  L  N  V  G  S  G  Y  N  E  F  N  K
AAAGTCCGGGCAAGTGACCATTTTCCGTTGGGGCTTCCCCGGCAAAAACCG     561
  K  S  G  Q  V  T  I  F  R  W  G  F  P  G  K  N  R
CCGCATTGAGTTGATCAATAAAATTGCCGATGTCCAGGCGGTAAAGGCGGA     612
  R  I  E  L  I  N  K  I  A  D  V  Q  A  V  K  A  E
AATTAAAGAAGGCGTTAACCCCAAACGCTCCCTATACCTCAAAGTCAAACA     663
  I  K  E  G  V  N  P  K  R  S  L  Y  L  K  V  K  Q
GCGAAGAGATATTCCCCTCACCAGGGCAGGCCAACCCATTTCGCTCTCCCA     714
  R  R  D  I  P  L  T  R  A  G  Q  P  I  S  L  S  Q
ATTGGAAAACCAGGGGGCTGAGTTAGCCCGCTTTCTAGGAGTACCCTAGAA     765
  L  E  N  Q  G  A  E  L  A  R  F  L  G  V  P  -
GGTTTGTAAATTGCCATTCCCGTGCTGTCAGATTTCACTACCAAGAACACC     816
AAATTCAGGGTCAGAAACGGGGGTTTGTTATGTTCAATTGGTCCCCATTTC     867
CCCTAGAAAGTTATCTAAAGTGGACTAAGGTTCCTAATAAGGACTTTAAAG     918
TTGGTTAATTTACATAACGATGAAGGCAGTTGCCATTACGAATTACGGAGT     969
TAGATTTTATGAGGATTTTACCGATATC                            997
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Figure 2. Genomic Sequence of *Synechocystis orf184.*

The nucleotide and deduced amino acid sequences of *Synechocystis orf184* are shown. The nucleotide sequence is shown from the HpaI site to the EcoRV site. The underlined sequence indicates a putative ribosome binding site. Boldface lettering indicates the start codon.

found from nine to four nucleotides upstream of the *orf184* start codon. The putative gene product would comprise 184 amino acids with a calculated mass of 21.5 kD and an isoelectric point of 10.75.

The deduced amino acid sequence of *Synechocystis orf184* was compared by multiple alignment (Figure 3) of corresponding sequences from the chloroplast genomes of tobacco (Shinozaki et al., 1986), rice (Hiratsuka et al., 1989), liverwort (Ohyama et al., 1986), and *E. gracilis* (Montandon et al., 1987). The cyanobacterial ORF184 protein is more similar to ORFs of the chloroplasts of higher plants (43 to 45% identity) than to that of *Euglena* (37%). Hydropathy plots show two highly hydrophobic domains typical of membrane proteins that could span the membrane in an α-helix conformation. These putative membrane-spanning regions are conserved in all reported *orf184* sequences.

Inactivation of the *orf184* in *Synechocystis*

To understand more about the functional role of the gene product, strains with inactivated *orf184* were generated. DNA gel blots of *Synechocystis* sp PCC 6803 DNA digested by restriction enzymes SphI, BamHI, EcoRI, and HindIII (data not shown)

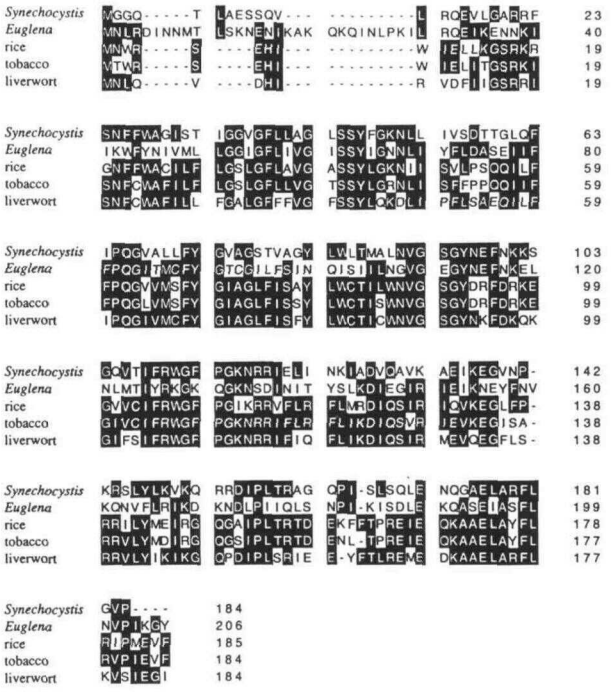

Figure 3. Comparison of the Deduced Amino Acid Sequences of *orf184*.

The deduced amino acid sequence of *Synechocystis orf184* is aligned with the corresponding ORFs from tobacco (*orf184*), rice (*orf185*), liverwort (*orf184*), and *Euglena* (*orf206*). Dashes indicate insertions or deletions introduced to maximize sequence identity.

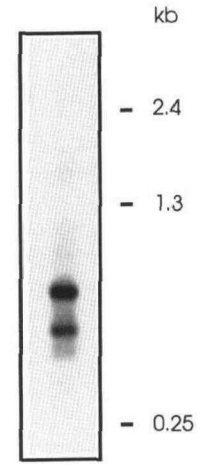

Figure 4. RNA Gel Blot Analysis of Wild-Type *Synechocystis* sp PCC 6803.

RNA was isolated from mid-log-phase cultures of wild-type cells and hybridized with an *orf184*-specific RNA probe to detect sense transcripts. Fifteen micrograms of total RNA was loaded per lane. Molecular length markers are given at right (RNA length standards were from Gibco-BRL, Eggenstein, Germany).

suggested that only a single copy of *orf184* exists in its genome. Before conducting mutagenesis, we investigated whether an *orf184* transcript could be detected in *Synechocystis* sp PCC 6803. A single-stranded *orf184*-specific RNA probe was found to hybridize to two transcripts of ~780 and 600 bp. These transcripts would be long enough to cover the entire coding region (Figure 4). No antisense transcripts were detected (data not shown). Because the 600-bp transcript was detected with a gene-specific single-stranded RNA probe, we explain this additional band as a specific degradation product of the 780-bp transcript. Importantly, the *orf184* probe did not hybridize to any higher molecular mass RNA species. This is in contrast to the situation in chloroplasts of rice and tobacco in which *orf184* has been shown to be cotranscribed with the gene encoding the apoprotein of cytochrome *f* (*petA*) (Shinozaki et al., 1986; Kanno and Hirai, 1993). Thus, it is very unlikely that the inactivation of the *orf184* sequence has polar effects on transcription of sequences located downstream of this ORF or on the opposite strand.

The strategy for inactivation of the ORF is outlined in Figure 1. Primary heterozygous transformants grow very well under photoautotrophic and photoheterotrophic conditions. Therefore, the rise of compensatory mutations at high frequency should be extremely low. Disruption or deletion of the *orf184* gene within the chromosome was confirmed by DNA gel blot analyses (Figure 5) using a 363-bp PstI-RsaI probe containing most of the *orf184* sequence. One of three transformants with a disrupted *orf184* sequence (14-1) and one of two deletion mutants (HM-1), which were selected on the basis of their resistance to kanamycin, were chosen for further studies. Additional hybridization experiments with HM-1 and

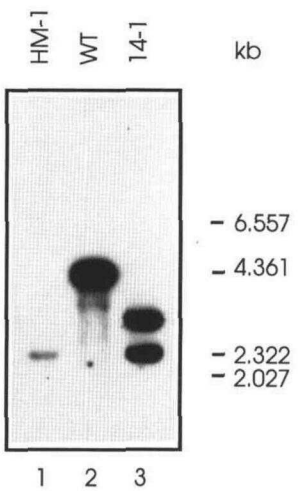

Figure 5. DNA Gel Blot Analysis of the Wild Type and *orf184* Mutants.

Gel blot analyses of HindIII-digested genomic DNA isolated from the *Synechocystis* sp PCC 6803 wild type (WT; lane 2) and *orf184* mutants 14-1 (lane 3) and HM-1 (lane 1) are shown. The intragenic 363-bp PstI-RsaI *orf184* fragment (Figure 2) was used as a probe. Insertion of the kanamycin resistance gene resulted in 3.3- and 2.3-kb fragments as compared with 4.3 kb in the wild type. Replacement of an HpaI fragment with the kanamycin resistance gene in mutant HM-1 resulted in the expected band of 2.2 kb. Molecular length markers are given at right in kilobases.

14-1 chromosomal DNA carrying the kanamycin resistance gene cartridge as a probe demonstrated its presence in fragments of the appropriate and predicted lengths (data not shown).

Under photoautotrophic and photoheterotrophic conditions, the growth rates of mutants 14-1 and HM-1 were essentially identical to that of wild-type *Synechocystis* sp PCC 6803 (data not shown).

Characteristics of the Photosynthetic Apparatus

Absorption spectra of intact cells of wild-type *Synechocystis* sp PCC 6803 and its *orf184* mutants 14-1 and HM-1 are presented in Figure 6. As is apparent from the spectra, there is a significant increase in the peak at 628 nm, reflecting phycobiliprotein absorbance, and a decrease in the peak at 681 nm, originating from chlorophyll (Chl) absorbance, for mutant strains 14-1 and HM-1 in comparison with the wild type. Interestingly, the 438-nm absorption maximum of Chl *a* was decreased significantly only in HM-1 and was virtually unaffected in mutant strain 14-1, suggesting possible differences in pigment assembly for both mutants. This contention is further supported by a greater content of Chl per cell for mutant 14-1 than for mutant HM-1 (Table 1), as well as by the relatively more drastic increase in phycobiliprotein absorbance for 14-1 (Figure 6, dotted line). According to the equations of Myers et al. (1980), the phycocyanin (PC)-to-Chl ratios (the ratio of A_{628}/A_{681}) are 1.5 (mutant 14-1), 1.3 (mutant HM-1), and 0.75 (wild type). Thus, these spectra show clearly that the PC/Chl ratio is substantially higher in both mutant strains than in wild-type cells. This leads to a pronounced "blue" phenotype for mutant strains when compared with the wild type.

The rate of light-saturated whole-chain electron transfer to the physiological electron acceptor CO_2 and the rate of the PSI- and PSII-mediated electron transfer reactions were between two- and threefold higher in the two mutant strains than in the wild type on the basis of Chl content (Table 2). These differences in electron transfer rates decreased when related to cell number because of the reduced Chl content in mutants, but remained significantly higher in mutants than in the wild type.

The high photochemical yield of PSII (indicated by oxygen evolution) in cells of the mutants as compared with wild-type cells was also evident from the increase in the variable maximum fluorescence yield (F_v) to maximum fluorescence yield (F_m) ratios measured by Chl *a* fluorescence (Table 3). The F_v/F_m ratio is an estimate of the photochemical efficiency of PSII when all reaction centers are in an open configuration. Its increase in the mutant strains was the result of an increase in both the dark-level fluorescence yield (F_o) and the F_m level. The F_o level in the mutants HM-1 and 14-1 was ~210 and 250%, respectively, higher than that observed in the wild type at the same Chl concentration. An increase in the F_o level is usually observed in PSII mutants with a decreased PSII/Chl ratio (e.g., Vermaas et al., 1994). In the case of strains HM-1 and 14-1, that should be the result of higher phycobiliprotein

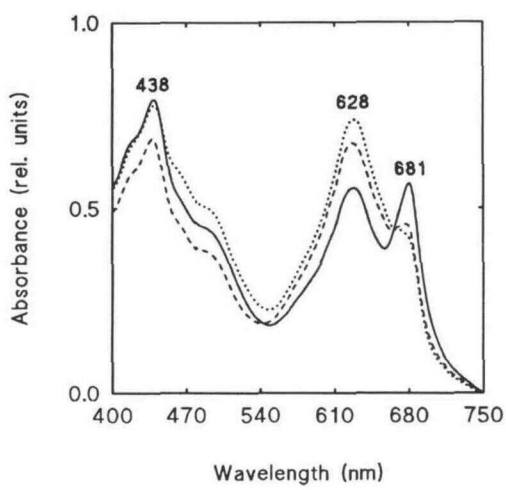

Figure 6. Absorption Spectra of the Wild Type and *orf184* Mutants.

In vivo absorption spectra were measured using whole cells of wild-type *Synechocystis* sp PCC 6803 (solid line) and its *orf184* mutants 14-1 (dotted line) and HM-1 (dashed line). The peaks at 438 and 681 nm mark the maxima of Chl *a* absorption, and the peak at 628 nm reflects the absorption maximum of PC. The spectra were corrected for light scattering at 750 nm.

Table 1. Pigment Content, Herbicide Binding, and EPR Measurements of $P700^+$ of Wild-Type *Synechocystis* sp PCC 6803 and Its *orf184* Mutants HM-1 and 14-1

Parameter	Wild Type	HM-1	14-1
Chlorophyll content (nmol Chl/10^6 cells)	9.3	5.2	6.0
PC/Chl ratio[a]	0.75	1.3	1.5
Chl/PSII[b]	450	230	170
EPR signal I ($P700^+$)[c]	1.0 (1.0)[d]	0.83 (0.46)	0.84 (0.55)

All values represent the means of at least four independent experiments.

[a] Determined from intact cells according to Myers et al. (1980).

[b] Determined using a ^{14}C-atrazine binding assay.

[c] Relative signal intensity.

[d] Values within parentheses were calculated on a per cell basis.

content per PSII center as reflected by the increased PC/Chl ratio. Moreover, the considerably higher F_m values, together with the finding that the absolute amplitude of F_v in mutants is ~400% of that found in wild-type cells, strongly indicate the presence of more photochemical active PSII centers per Chl in mutant strains than in the wild type. Such a conclusion can also be drawn from the 77 K fluorescence emission spectra presented in Figure 7. These spectra were obtained by excitation of the *Synechocystis* sp PCC 6803 wild type and *orf184* mutants 14-1 and HM-1 at 440 nm (the maximum of the Soret absorption band for Chl a). Three characteristic peaks at 685 and 695 nm, reflecting fluorescence emission from PSII Chl *a*, as well as at 725 nm, originating predominantly from PSI Chl *a*, are evident. A dramatic increase in the PSII/PSI fluorescence emission ratio becomes obvious for both mutant strains; the ratio is somewhat higher for strain 14-1.

The results obtained with fluorescence measurements indicated a strong increase in the number of PSII reaction centers in mutants as compared with the wild type. To quantify the amount of PSII centers in vivo, binding assays were performed with different concentrations of radiolabeled atrazine. The number of PSII units in intact cells can be quantified on a Chl basis using ^{14}C-atrazine, which binds specifically to the D1 protein of a physiologically functional PSII unit. Figure 8 shows double-reciprocal plots for the binding of ^{14}C-atrazine in cells of wild-type *Synechocystis* 6803 and *orf184* mutants 14-1 and HM-1. There is a strong decrease in the Chl/PSII ratio in both mutant genotypes when compared with the wild type. Calculated Chl/PSII ratios of wild-type and mutant strains (Table 1) imply an increase in the number of PSII centers by factors of ~2.0 (mutant HM-1) and 2.65 (mutant 14-1). It is also apparent from Figure 8 that atrazine binding affinity was not affected in mutants. Room temperature electron paramagnetic resonance (EPR) spectroscopy was used to detect light-induced EPR signal I, which originates from $P700^+$ formation (Hoff, 1987), in whole cells from mutant and wild-type strains. Although the total amount of PSI reaction centers in the wild type and mutants cannot be quantified by room temperature EPR spectroscopy of $P700^+$ formation, statements on relative changes in the number of PSI reaction centers are possible. Thus, the relative decrease in the magnitude of signal I obtained with cells of the mutants compared with wild-type cells after illumination with saturating light in the presence of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) reflects a decrease in the number of PSI reaction centers (Table 1). Taking into account the strongly decreased Chl content in the mutants, the decrease in the amount of PSI reaction centers is even more prominent on a per cell basis. It should be mentioned that there were no alterations in the spectral characteristics of the EPR signals in mutant strains.

The increase in the number of PSII centers, accompanied by the decline in the amount of PSI reaction centers per Chl, results in 2.4- (HM-1) and 3.1-fold (14-1) higher PSII/PSI ratios in mutants than in the wild type. This finding agrees with the results of 77 K fluorescence measurements, indicating drastically altered PSII/PSI ratios in both mutants (see Figure 7).

Furthermore, we reverted the mutants to the wild-type phenotype. The HM-1 deletion strain was transformed with a plasmid containing *orf184* and a chloramphenicol resistance gene cartridge. The selected chloramphenicol-resistant transformants lost their kanamycin resistance and had the green appearance of wild-type cells. Complete replacement of the mutant copy by wild-type *orf184* was confirmed by DNA gel blot analysis and led to 77 K fluorescence emission and absorption spectra identical to those of the wild type (data not shown). These results demonstrate that the phenotype of mutants HM-1 and 14-1 is due to the inactivation of *orf184* rather than to a compensatory mutation.

DISCUSSION

All completely sequenced chloroplast genomes, except that of the nonphotosynthetic parasitic plant *Epifagus virginiana* (Wolfe et al., 1992b), contain the conserved *orf184/185* (also

Table 2. Rates of Electron Transfer Reactions in Cells

Section	Wild Type	HM-1	14-1
PSI (DCPIP/Asc:MV)	31.0 (3.0)	84.5 (4.4)	87.4 (4.9)
PSII (H_2O: PQ/$K_3Fe(CN)_6$)	58.2 (5.8)	111.8 (6.7)	136.8 (7.8)
PSI + PSII (H_2O: $NaHCO_3$)	185.1 (17.4)	417.5 (22.5)	459.1 (26.3)

All values represent the means of at least four independent experiments. Values are based on micromoles of oxygen per milligram Chl per hr or per 10^6 cells per hr (within parentheses). Asc, ascorbate; DCPIP, 2,6-dichlorophenol-indophenol; MV, methyl viologen; PQ, *p*-benzoquinone.

Table 3. Room Temperature Fluorescence Parameters (Relative Units)

Parameter	Wild Type	HM-1	14-1
F_o	20.1	43.0	52.2
F_m	40.2	125.1	136.4
F_v	20.1	82.1	84.2
F_v/F_m	0.49	0.66	0.62

All values represent the means of at least four independent experiments. Samples were adjusted to a Chl concentration of 20 μg mL^{-1}.

designated *ycf4* for "hypothetical chloroplast open reading frame 4," according to new nomenclature; Hallick et al., 1993). No other sequence similarities with already known genes have been identified. We used a reverse genetics approach to obtain *orf184* mutants of the cyanobacterium *Synechocystis* sp PCC 6803 and characterized these mutants. We succeeded in cloning *orf184* of the cyanobacterium using *orf184* of tobacco as a heterologous probe. The amino acid sequence similarities between the cyanobacterial and the chloroplast *orf184* genes are 46, 45, 43, and 37% for tobacco, liverwort, rice, and *Euglena gracilis*, respectively. These values are much lower than those reported for essential photosynthetic genes: for example, *psbA* (Mulligan et al., 1984), *psaA* and *psaB* (Cantrell and Bryant, 1987), and *psaD* (Reilly et al., 1988). But there are some domains with high sequence identity values, indicating that these parts of the sequence have to be conserved to retain the function of the protein. Furthermore, the hydrophilicity–hydrophobicity profiles for the *orf184* gene products of *Synechocystis* sp PCC 6803 and chloroplasts are very similar. They indicate that the proteins contain two transmembrane α-helices, suggesting their localization within the thylakoid or cell/plastid membrane.

It has recently been demonstrated that *orf185* is cotranscribed with *orf230* and *petA* in rice chloroplasts (Kanno and Hirai, 1993). Our preliminary studies on the transcription of *orf184* in *Synechocystis* sp PCC 6803 suggest that it is not part of an operon. Also, the location within the genome differs between chloroplasts and cyanobacteria. *orf184* has been located on a physical and genetic map of the *Synechocystis* sp PCC 6803 genome ~400 kb from the operon coding for large and small subunits of ribulose bisphosphate carboxylase (*rbcL* and *rbcS*; Churin et al., 1995). In contrast *orf184* is located close to *rbcL* in the chloroplast genomes of higher plants (Ohyama et al., 1986; Shinozaki et al., 1986; Hiratsuka et al., 1989).

The conserved amino acid sequence of *orf184* across relatively large phylogenetic distances suggests an important role in chloroplast and cyanobacterial cell function. To address this role, we inactivated the *orf184* gene. We were able to select *orf184* mutants that grew photoautotrophically and photoheterotrophically. Phenotypically, they differed from the wild type by being more bluish in color. Surprisingly, when *orf184* was inactivated, there was no effect on the growth rates of the HM-1 and 14-1 mutant strains. Although this finding demonstrates that the *orf184* gene product is not essential for growth of *Synechocystis* sp PCC 6803 cells under the current experimental conditions of cultivation, additional analyses of *orf184* mutants are needed to provide evidence that the protein plays a significant role in photosynthetic processes.

The *Synechocystis* sp PCC 6803 *orf184* mutants are characterized by an increase in the rates of photosynthetic electron transfer between two- and threefold higher than that of wild-type cells when expressed on the basis of Chl content. This increase can be explained in terms of extensive changes in the structural organization of the photosynthetic apparatus. In *Synechocystis* cells, phycobilisomes are associated with PSII, whereas most of the Chl is associated with PSI (Bryant, 1992). In mutant cells, there was a 44% (HM-1) and 35% (14-1) reduction in Chl content. Inversely, an increase in the phycobilin pigment content was found in the mutants. The increase in the ratios of PC/Chl was paralleled by an increase in the PSII/PSI ratios. The latter was deduced by 77 K fluorescence, herbicide binding, and EPR measurements of $P700^+$ formation. Mutant strains contain nearly two (HM-1) and three (14-1) times more PSII centers but only ~80 to 85% of the PSI centers, on the basis of Chl content, when compared with the wild type.

Thus, the enhanced PSII electron transfer rates in both mutant strains on the basis of Chl content coincide reasonably well with the apparent increase in the amount of PSII. This also indicates that the accumulated PSII centers are functionally intact. Another situation was observed for PSI. Here, the increase in the electron transfer activity is difficult to reconcile

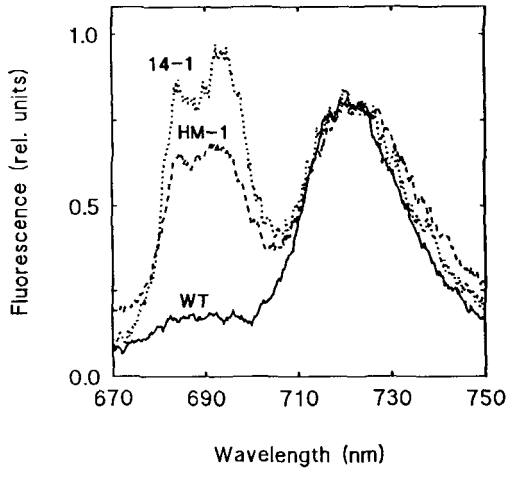

Figure 7. The 77 K Fluorescence Emission Spectra after Excitation at 440 nm.

The 77 K fluorescence emission spectra of whole cells of wild-type (WT) *Synechocystis* sp PCC 6803 (solid line) and its *orf184* mutants 14-1 (dotted line) and HM-1 (dashed line) after excitation at 440 nm (Chl *a* excitation). Cells were adjusted to a Chl concentration of 2.5 μg mL^{-1}. The spectra were normalized to the emission maximum at 725 nm.

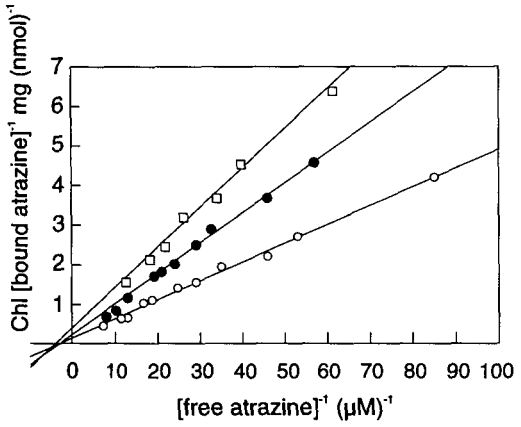

Figure 8. Double-Reciprocal Plots of Atrazine Binding.

^{14}C-Atrazine binding to whole cells of wild-type *Synechocystis* sp PCC 6803 (□) and its *orf184* mutants 14-1 (○) and HM-1 (●). ^{14}C-Atrazine was added at various concentrations to 1-mL samples in 25 mM Hepes-NaOH, pH 7.0. The Chl concentration in each sample was 25 μg mL^{-1}.

only with the decrease in PSI content. This discrepancy could result from the use of Chl as a reference value. Because the PSI antenna contains more Chl *a* molecules (~65 to 130 Chl; Williams et al., 1983; Rögner et al., 1990; Witt et al., 1990) than does the PSII antenna (between 35 and ~50 Chl; Satoh, 1983; Glazer and Melis, 1987), Chl content is a variable component when PSII and/or PSI composition is changed. Moreover, Shen and Vermaas (1994) recently discussed the existence of Chl binding proteins other than those known to be associated with the PSII and PSI cores. Thus, the Chl content might not necessarily be an adequate basis for expressing electron transfer rates. Indeed, when calculating the rates on a per cell basis, the differences between mutant and wild-type cells became smaller. This implies that the number of PSI centers is not a limiting factor for PSI electron transfer in cells of the *orf184* mutants. Furthermore, it suggests that factors other than P700 are responsible for the increase in PSI electron transfer activities. Additional investigation is required to determine whether the mutation affects PSI assembly, which might lead to the observed phenotype.

An increase in the PC/Chl ratio was reported after growth of *Anacystis* in high light in the presence of sublethal concentrations of DCMU-type inhibitors (Koenig, 1990). This phenomenon was shown to be not simply the consequence of an imbalance in electron transport. Koenig (1990) assumed that the adaptive reorganization of the photosynthetic apparatus was controlled at the level of the D1 protein.

Changes in the stoichiometry of the two photosystems have been shown to depend on the light intensity and quality in thylakoids of chloroplasts (Chow et al., 1990; Melis, 1991) and in cyanobacteria (Fujita and Murakami, 1987; Aizawa et al., 1992; Murakami and Fujita, 1993). This indicates that the overall adjustment of a respective PSII/PSI ratio is a common ability to regulate light capture. Recent studies with cyanobacteria have demonstrated that PSI is the variable component in changes in PSII/PSI stoichiometry in response to the light regime for photosynthesis and that the PSII level remains rather constant (Fujita and Murakami, 1987; Aizawa et al., 1992; Samson et al., 1994). The increase in the PSII/PSI ratio found in our study was the result of both an increase in the PSII level and a decrease in the PSI level, that is, the mutants exhibit a unique, hitherto undescribed phenotype. As yet, we have no explanation for the observed differences between mutants HM-1 and 14-1. It should be noted that in the case of HM-1, a deletion should lead to the complete absence of the ORF184 protein, whereas its N-terminal part could still be present in cells of the 14-1 mutant.

In conclusion, the data presented here show that inactivation of a *Synechocystis* sp PCC 6803 gene with a striking homology to the tobacco chloroplast *orf184* gene increases rates of photosynthetic electron transfer and results in a higher PSII/PSI ratio in the thylakoid membrane, thereby suggesting a functional role for the *orf184* gene product in the structural organization of the photosynthetic membrane and in the adjustment of the PSII/PSI ratio.

METHODS

Growth Conditions

Synechocystis sp strain PCC 6803 wild-type and mutant strains were grown at 30°C in BG-11 medium (Rippka et al., 1979). Cells were grown at a light intensity of ~40 μE m^{-2} sec^{-1}. Transformants of *Synechocystis* sp PCC 6803 were selected on media with increasing amounts of kanamycin (10 to 50 μg mL^{-1}). Growth of the wild-type and mutant strains was followed under photoautotrophic and photoheterotrophic (5 mM glucose, 5 μE m^{-2} sec^{-1}) conditions by monitoring the optical density at 750 nm (DU50 spectrophotometer; Beckman, Glenrothes, Scotland) and the cell number by light microscopy (hemocytometer; Thoma, Bad Blankenburg, Germany).

DNA Isolation and Hybridization Procedures

Genomic DNA of *Synechocystis* sp PCC 6803 was extracted as described by Franche and Damerval (1988). After digestion of DNA by restriction endonucleases and electrophoresis, gel blotting to nylon membranes (Hybond N; Amersham, Braunschweig, Germany) was performed. DNA probe fragments were labeled with α-^{32}P-dATP (Amersham) by the random priming method as recommended by the manufacturer. DNA gel blot hybridizations were performed as described by Sambrook et al. (1989), with a PstI-RsaI fragment of *Synechocystis* DNA extending from nucleotides 393 to 756 (see Figure 2).

RNA Gel Blot Analysis

Total cellular RNA was isolated from cells according to the method described by Logemann et al. (1987). The 363-bp PstI-RsaI fragment containing most of the open reading frame *orf184* sequence was subcloned into pBluescript KS+ (Stratagene), from which sense and

antisense RNA probes were transcribed using a T3/T7 RNA transcription kit (Ambion, Austin, TX). The probe generated by transcription with T3 RNA polymerase was used to detect *orf184* transcripts, and the probe generated by transcription with T7 RNA polymerase was used to detect antisense transcripts.

Cloning and Sequence Analysis

Restriction endonucleases were obtained from Amersham and used according to the manufacturer's recommendations. The sequence homologous to chloroplast *orf184* was identified in a λ EMBL3 genomic library of *Synechocystis* sp PCC 6803 (kindly provided by M. Hagemann, University of Rostock, Rostock, Germany), using an *orf184*-specific EcoRV fragment (nucleotides 62775 to 63195) of clone pTB22 from a tobacco chloroplast gene library (Sugiura et al., 1986) as a heterologous probe. A HindIII fragment bearing the cyanobacterial *orf184* was cloned in the pBluescript KS+ vector (subclone pkH1). Nucleotide sequences were aligned using PC/Gene (IntelliGenetics, Inc./Betagen, Mountain View, CA) on an IBM PC–compatible computer and using Husar-GCG software on a CONVEX C3820 computer (DKFZ, Heidelberg, Germany).

The nucleotide sequence of a 1484-bp region of the *Synechocystis* sp PCC 6803 genome containing *orf184* has been submitted to EMBL as accession number Z27404.

Inactivation of the *Synechocystis orf184*

The pkH1 subclone was used to construct cyanobacterial mutants (Figure 1). A 1.2-kb EcoRI and a 1.3-kb PvuII fragment from pUC4K served as a kanamycin resistance gene cartridge (Vieira and Messing, 1982). *Synechocystis* cells were then transformed with 1 μg of DNA from recombinant plasmids, following the protocol described by Ermakova et al. (1993). After growth in liquid medium for 1 hr, the cells were spread on nonselective plates. Cells were grown for 10 hr, and plates were then underlayered with kanamycin (5 μg mL^{-1}). Homozygous mutants (confirmed by DNA gel blot analysis) were obtained after five serial streak purifications of single colonies on plates containing increasing concentrations of kanamycin (final concentration 50 μg mL^{-1}).

One of the selected mutant strains (HM-1 with deleted *orf184*) was transformed back to the wild-type phenotype with a plasmid containing the wild-type copy of *orf184* and a chloramphenicol resistance gene cartridge (a 1.4-kb BsaAI fragment of pACYC184; New England Biolabs, Beverly, MA) 220 bp upstream of the *orf184* start codon. The construct contained sequences that permitted its incorporation into the genome by homologous recombination, thereby replacing the previously inserted kanamycin resistance gene. Transformants were selected on 7 μg mL^{-1} chloramphenicol.

Photosynthetic Electron Transport Assays

Oxygen evolution from whole cells suspended in a buffer solution of 25 mM Hepes-NaOH, pH 7.0, was measured polarigraphically with a Clark-type electrode at a chlorophyll (Chl) concentration of 5 μg mL^{-1}. For measurement of whole-chain electron transfer from H_2O to CO_2, 10 mM $NaHCO_3$ was added to the reaction medium. Photosystem II (PSII)–mediated electron transfer was measured in the presence of 0.5 mM $K_3Fe(CN)_6$ and 0.25 mM *p*-benzoquinone. Photosystem I (PSI)–mediated electron transfer was measured in thylakoid membranes by monitoring oxygen consumption after the addition of 2 mM sodium ascorbate, 40 μM 2,6-dichlorophenol-indophenol, 20 μM methyl viologen, 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and 1 mM KCN. The electrode chamber temperature was maintained at 30°C. The chamber contents were continuously stirred during the experiments. Saturating white, heat-filtered actinic light (520 μE m^{-2} sec^{-1}) was provided by a KL 1500 light source (Schott, Mainz, Germany). Chl contents were measured by extraction of whole cells in 90% methanol using the method of MacKinney (1941).

Herbicide Binding Analysis and $P700^+$ Determination

Binding assays of ^{14}C-atrazine to intact cells of wild-type *Synechocystis* sp PCC 6803 and its *orf184* mutants 14-1 and HM-1 were performed as described previously (Vermaas et al., 1990). Cells were collected at an optical density of ~0.6 to 0.7 at 750 nm and resuspended to 25 μg mL^{-1} Chl in 25 mM Hepes-NaOH, pH 7.0. Aliquots were incubated in dim light for 30 min at room temperature. The total amount of atrazine binding to the cells was determined by measuring the content of ^{14}C-atrazine in the supernatant and, to improve the accuracy, also in the pellet.

Electron paramagnetic resonance (EPR) *spectra of the P700 signal* (photo-oxydized 700) were recorded at room temperature, using an ECS 106 X-band spectrometer (Bruker Instr., Rheinstetten, Germany) equipped with a high-sensitivity rectangular-mode cavity ER 4102ST. The instrumental parameters are as follows: microwave power, 20 mW; modulation amplitude, 0.4 millitesla; time constant, 100 msec. Whole cells were adjusted to a Chl concentration of 1 mg mL^{-1} in 25 mM Hepes-NaOH buffer, pH 7.5, supplemented with 7.5% polyethylene glycol 4000, 5 mM EDTA, and 100 μM DCMU (final concentration). Illumination (1000 μE m^{-2} sec^{-1}) was provided by a Schott KL 1500 light source. Before each measurement, samples were dark adapted for 10 min.

Absorption, 77 K, and Room Temperature Fluorescence Emission Measurements

In vivo absorption spectra of whole cells of wild-type *Synechocystis* sp PCC 6803 and its *orf184* mutants 14-1 and HM-1 suspended in BG-11 medium were measured at room temperature using a UVIKON 931 spectrophotometer (Kontron, Milan, Italy). The phycocyanin (PC)/Chl ratios were calculated from the absorption spectra using the equations of Myers et al. (1980).

Low-temperature fluorescence emission spectra (77 K) were recorded using an F-4500 spectrophotometer (Hitachi, Tokyo, Japan). Cell suspensions were diluted to a concentration of 2.5 μg mL^{-1} Chl in 25 mM Hepes-NaOH, pH 7.0. After 10-min darkening, samples were rapidly frozen in liquid nitrogen. The excitation and emission slitwidths were 5 and 2.5 nm, respectively.

Room temperature Chl *a* fluorescence was measured with a pulse amplitude modulation Chl fluorometer (Walz, Effeltrich, Germany), following the protocol of Clarke et al. (1993). Actinic and measuring light were provided by red light–emitting diodes, with an emission maximum around 650 nm (half bandwidth, 25 nm). For measurements, samples were concentrated to a Chl content of 20 μg mL^{-1}. All experiments were performed at 30°C. A KS 101 suspension cuvette (Walz) was used to maintain a constant sample environment during measurement. Dark-level fluorescence (F_o) was determined by illuminating 10-min dark-adapted cells with modulated light with an intensity of ~0.05 μE m^{-2} sec^{-1}. After a stable F_o level was obtained, cells were illuminated by actinic light with a photon flux density of 50 μE m^{-2}

sec^{-1} until a stable fluorescence yield was observed. The maximum fluorescence yield (F_m) was then determined by injecting 10 μM DCMU into the cuvette; the resulting level was taken to be F_m. Maximum variable fluorescence (F_v) was calculated as the difference between the F_m level obtained after addition of DCMU and the F_o level in the dark-adapted state.

Photon flux density was measured with a quantum sensor (LI-189A; LI-COR, Lincoln, NE).

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