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The Sll0606 Protein Is Required for Photosystem II Assembly/Stability in the Cyanobacterium *Synechocystis* sp. PCC 6803*

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An insertional transposon mutation in the *sll0606* gene was found to lead to a loss of photoautotrophy but not photoheterotrophy in the cyanobacterium *Synechocystis* sp. PCC 6803. Complementation analysis of this mutant (*Tsll0606*) indicated that an intact *sll0606* gene could fully restore photoautotrophic growth. Gene organization in the vicinity of *sll0606* indicates that it is not contained in an operon. No electron transport activity was detected in *Tsll0606* using water as an electron donor and 2,6-dichlorobenzoquinone as an electron acceptor, indicating that Photosystem II (PS II) was defective. Electron transport activity using dichlorophenol indolephenol plus ascorbate as an electron donor to methyl viologen, however, was the same as observed in the control strain. This indicated that electron flow through Photosystem I was normal. Fluorescence induction and decay parameters verified that Photosystem II was highly compromised. The quantum yield for energy trapping by Photosystem II (F_V/F_M) in the mutant was less than 10% of that observed in the control strain. The small variable fluorescence yield observed after a single saturating flash exhibited aberrant Q_A^- reoxidation kinetics that were insensitive to dichloromethylurea. Immunological analysis indicated that whereas the D2 and CP47 proteins were modestly affected, the D1 and CP43 components were dramatically reduced. Analysis of two-dimensional blue native/lithium dodecyl sulfate-polyacrylamide gels indicated that no intact PS II monomer or dimers were observed in the mutant. The CP43-less PS II monomer did accumulate to detectable levels. Our results indicate that the Sll0606 protein is required for the assembly/stability of a functionally competent Photosystem II.

In higher plants, algae, and cyanobacteria, at least six intrinsic proteins appear to be required for oxygen evolution by PS II² (1–3). These are CP47, CP43, the D1 and D2 proteins, and the α and β subunits of cytochrome b_{559} . Insertional inactivation or deletion of the genes for these components results in the absence of PS II complex assembly and the complete loss of oxygen evolution activity (for a review, see Ref. 4). For maximal

rates of oxygen evolution in cyanobacteria, the extrinsic proteins PsbO, PsbU, PsbV, and PsbQ must also be present (5). Additionally, a large number of other intrinsic membrane components are present in PS II complexes (6–8), although the functions of many of these proteins remain obscure. The most recent crystal structure of the thermophilic cyanobacterium *Thermosynechococcus elongatus* (9) indicates that PS II contains 20 protein components (it should be noted that PsbQ, which is essential for maximum PS II activity in cyanobacteria (10), is missing from the current crystal structure).

PS II assembly and turnover requires a variety of other protein components (for a comprehensive review, see Ref. 11). Although many of these proteins are conserved in all oxygenic organisms, a subset is present only in the cyanobacteria. These include the *Synechocystis* sp. PCC 6803 (henceforth *Synechocystis*) proteins Slr0286 (12) and Slr2013 (13), which were identified during the screening of suppressor strains of various D2 mutants. The functions of these components remain poorly understood. The PrtA protein (encoded by *slr0248*) is a periplasmic component that appears to be involved in D1 processing (14) and may directly interact with the C-terminal domain of the D1 protein (15). Finally, an operon containing six genes (*slr0144*–*slr0152*) has been identified, which may be involved in the assembly/stability of PS II (16). The deletion of this operon leads to a 35% loss of oxygen evolution, slower deactivation of the higher S-states, and slower photoautotrophic growth. It should also be noted that carotenoids (as well as chlorophyll) are critically important for PS II assembly/stability. Simultaneous genetic deletion of the *crtB* and *crtH* genes in *Synechocystis* leads to a complete loss of carotenoid biosynthesis and the loss of assembly of intact, fully functional PS II reaction centers. This is the result of a markedly decreased accumulation of the CP47, CP43, and D1 proteins under either light-activated heterotrophic or continuous illumination growth conditions. The small amounts of CP47 and D1 that do accumulate are found almost exclusively in the CP43-less RC47 complex (17), with little or no assembled CP43 being observed. Consequently, carotenoids appear to be required for CP47, CP43, and D1 accumulation and integration into the functional PS II reaction center complex.

In vitro transposon mutagenesis is a powerful tool for identifying genes required for photoautotrophy (18). Earlier, we have used this technique to identify previously undescribed components of the cyanobacterial carbon-concentrating mechanism (19) and have also elucidated a differential role for the malic enzyme in carbon metabolism under continuous *versus* diurnal illumination (20). In the current study, a cyanobac-

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² The abbreviations used are: PS I and II, Photosystem I and II, respectively; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LiDS, lithium dodecyl sulfate.

Sll0606 Is Required for PS II Assembly/Stability

terial mutant (designated *Tsll0606*) bearing a transposon insertion in the *sll0606* gene was isolated. This mutant exhibits drastically altered PS II characteristics and fails to integrate CP43, D1, and CP47 into functional oxygen-evolving PS II reaction centers. The characteristics of this mutant indicate that the Sll0606 protein is a cyanobacteria-specific assembly/stability factor for PS II.

MATERIALS AND METHODS

Cyanobacterial Strains and Growth Conditions—A glucose-tolerant strain of *Synechocystis* sp. strain PCC 6803 (21) was used as the DNA recipient strain in this study, and the derivative kanamycin-resistant strain K3 (22) was used as a wild-type control strain. Cells of all strains were maintained under photoheterotrophic growth conditions at 30 °C with a light intensity of 20 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ on BG-11 growth medium (23) supplemented with 10 mM TES-KOH (pH 8.2), 5 mM glucose, 10 μM *N*-(3,4-dichlorophenyl)-*N'*-dimethylurea (DCMU), 0.3% (w/v) sodium thiosulfate, and 1.5% (w/v) agar. Where appropriate, kanamycin was included in the medium at a final concentration of 10 $\mu\text{g}/\text{ml}$. For photoautotrophic growth, no glucose or DCMU was added to the medium. For liquid cultures, the agar and sodium thiosulfate were omitted, and the cultures were continuously bubbled with sterile, humidified air.

DNA Manipulation and *in Vitro* Transposon Mutagenesis—Genomic DNA of the recipient and mutant strains was prepared as described previously (18). Ten micrograms of the genomic DNA from the DNA recipient strain of *Synechocystis* was digested with the restriction enzyme HhaI or BfuCI. The cut DNA fragments were then incubated with the transposase enzyme and transposon DNA of the EZ-TN5 Kan-2 *in vitro* transposition system according to the manufacturer's instructions (Epicenter). Following the *in vitro* transposition reaction, the DNA fragments were used to transform the DNA recipient strain of *Synechocystis* cells. As described previously (18), the kanamycin-resistant transformants were grown photoheterotrophically for a period of about 4 weeks with weekly transfers in the presence of kanamycin and screened in a semi-high throughput manner for the ability to grow photoautotrophically. Upon plating onto standard BG-11 medium, the putative photoautotrophic mutant strains were identified as those that repeatedly failed to grow photoautotrophically in the absence of glucose (with kanamycin) but grew photoheterotrophically in the presence of glucose and DCMU (with kanamycin).

Inverse PCR—To identify the transposon insertion sites in the genome, inverse PCR was performed as described previously (18). In brief, 1–5 μg of the genomic DNA was digested with the restriction enzyme RsaI (New England Biolabs), and the fragments were then self-ligated with a T4 DNA ligase (Promega). The circularized fragments were used as templates in a PCR designed to amplify the DNA region containing the transposon insertion site. The transposon-specific primers TnR1 (5'-ACCATGAGTGACGACTGAATCCG-3') and TnF1 (5'-ACCTACAACAAAGCTCTCATCAACC-3') were used in this amplification. The PCR mixtures contained 200 μM dNTPs, 0.4–1.0 μM each primer, and 2.5 units of TaqDNA polymerase per 100- μl reaction in 1 \times reaction buffer (Takara Bio Inc.). The reactions were performed with 35–40 cycles of 94 °C for 30 s,

45–55 °C for 60 s, and 72 °C for 2–4 min. Upon separation by agarose gel electrophoresis, the PCR products were purified and subjected to DNA sequencing using the transposon-specific primers TnR1 and TnF1. Sequence homology searches were performed with the BLAST program (24).

Genetic Complementation—Complementation testing was performed by transforming *Tsll0606* mutant cells with the wild-type *sll0606* gene. Briefly, the wild-type DNA fragment of the *sll0606* gene was obtained through PCR amplification using the genomic DNA of *Synechocystis* as a template. A pair of primers, F1 (5'-GTGTTTGGTCGGTTGCCCTTA-3') and R1 (5'-TGCTAACTCAGCGGTTAATGG-3') (Fig. 1) were used to amplify a 1428-bp fragment of the full-length gene. This fragment was then introduced into the *Tsll0606* mutant cells by standard transformation procedures. Numerous colonies were identified which were able to grow photoautotrophically but only in the absence of kanamycin. Several of these strains were subjected to DNA sequencing, and it was confirmed that all had the wild-type *sll0606* gene restored and that the transposon was no longer present (data not shown).

Electron Transport Measurements— O_2 evolution rates were measured by polarography with a Hansatech oxygen electrode at 24 °C with a light intensity of 2000 μmol of photons $\text{m}^{-2} \text{s}^{-1}$. Cells of the K3 control strain and the *Tsll0606* mutant were grown photoheterotrophically and harvested during late exponential growth phase. After washing twice with BG-11 medium, the cells were assayed at a chlorophyll concentration of 10 $\mu\text{g}/\text{ml}$ in BG-11. Chlorophyll and carotenoid concentrations were determined using the methods of Lichtenthaler (25). PS II activity from H_2O to 2,6-dichloro-*p*-benzoquinone was measured in the presence of 1.0 mM 2,6-dichloro-*p*-benzoquinone. PS I-catalyzed electron transport activity was assayed with 10 μM DCMU, 0.1 mM 2,6-dichlorophenolindophenol, 5 mM ascorbate, 1.0 mM sodium azide, and 1.0 mM methyl viologen. All measurements were repeated at least three times, and the average O_2 evolution rates are presented in this study.

Fluorescence Measurements—Fluorescence induction and decay were monitored with a Photon Systems Instruments (Brno, Czech Republic) FL3000 dual modulation kinetic fluorometer (commercial version of the instrument described in Ref. 26). Both measuring and saturating flashes were provided by computer-controlled photodiode arrays. The flash profile exhibited a square shape for the low power measuring flashes and deviated only 5% from an ideal square shape for saturating actinic flashes. For all of the fluorescence experiments, cells were grown photoheterotrophically and harvested in the exponential growth phase. After washing twice with BG-11 medium, the cells were incubated at a chlorophyll concentration of 5 $\mu\text{g}/\text{ml}$ in the BG-11 medium in the dark for 5 min before initiation of the experiments.

In the PS II fluorescence induction experiments, data were collected in a logarithmic time series between 10 μs and 1 s upon onset of continuous illumination. Data were collected at a frequency of 10 MHz with 12-bit resolution. In the Q_A^- reoxidation experiments, a single saturating flash was provided at the onset of the experiment. The kinetics of the transfer of an electron between Q_A^- and Q_B were examined in the absence of DCMU (unnormalized data from this experiment were also

used to determine the fluorescence parameters presented in Table 1), whereas the charge recombination reactions between Q_A^- and PS II donor side components were examined in the presence of DCMU. For these experiments, data were collected between 10 μ s and 60 s following a single saturating flash. In the DCMU treatment experiments, the cells were incubated with 10 μ M DCMU for at least 5 min prior to initiation of the fluorescence experiments. Data were analyzed using Origin version 6.1 (Microcal Software Inc., Northampton, MA) and proprietary software provided by Photon Systems Instruments.

Immunological Characterization of Cyanobacterial Membrane Proteins—The cyanobacterial membranes (thylakoids and plasma membrane) were isolated from the control and *Tsll0606* cell lines by modification of the procedure previously described (27). Six-day-old cultures (450 ml) of photoheterotrophically grown cells were pelleted by centrifugation at 4 °C for 5 min at 2000 \times *g*. The cell pellet was resuspended in fresh BG-11 medium and again pelleted at 4 °C for 5 min at 2000 \times *g*. The cell pellet was then resuspended in Break Buffer containing 50 mM MES-NaOH, pH 6.0, 10 mM MgCl₂, 5 mM CaCl₂, and 25% glycerol and incubated for 1 h on ice. After incubation, the cells were loaded into a prechilled Bead-Beater chamber (Bio-Spec Products), and glass beads (0.1 mm) were added to give a 1:1 ratio of glass beads to cell suspension. The Bead-Beater chamber was cooled with a water-ice jacket. The cells were then broken with 10 break cycles, each cycle consisting of 10 s of homogenization followed by 2 min of cooling. After breakage, the cell homogenate was decanted from the glass beads, and the beads were washed twice with Break Buffer to recover additional homogenate. The homogenate was centrifuged at 2000 \times *g* for 5 min to remove unbroken cells and residual glass beads, and the supernatant was then centrifuged at 38,000 \times *g* for 30 min to pellet the cyanobacterial membranes. These were resuspended in a small volume of Break Buffer.

Analytical PAGE of the PS II proteins was performed under conditions described by Delepelaire and Chua (28) in gradient 12.5–20% polyacrylamide gels. The resolved proteins were electroblotted onto PVDF membranes (Immobilon-P, Millipore Corp.). After blocking for 2 h with 5% nonfat dry milk in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, the blots were washed extensively with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4. The blots were then incubated sequentially with diluted primary antibodies followed by incubation with an anti-rabbit IgG-peroxidase conjugate (Sigma) as the secondary antibody. The labeled proteins were detected using chemiluminescence (Pierce). The anti-PsbA, anti-PsbB, anti-PsbC, anti-PsbD, and anti-PsaA antibodies were obtained from Agrisera, whereas the anti-cytochrome *f* antibody was produced in house. The x-ray films were scanned with an Epson V700 color scanner at 600 dpi resolution and a 24-bit color depth. The ImageJ program (29) was used to analyze the scanned images.

Two-dimensional Blue Native/LiDS-PAGE—Blue native electrophoresis was performed as described by Cline and Mori (30) in a 5–13.5% linear polyacrylamide gradient gel. Samples were prepared as described by Herranen *et al.* (31) with solubilization in 2% dodecyl- β -D-maltoside and 10 μ g of chlorophyll loaded in each lane of the first dimension gel. Electrophoresis was performed for 5.5 h at 2 watts at 4 °C. After electrophoresis,

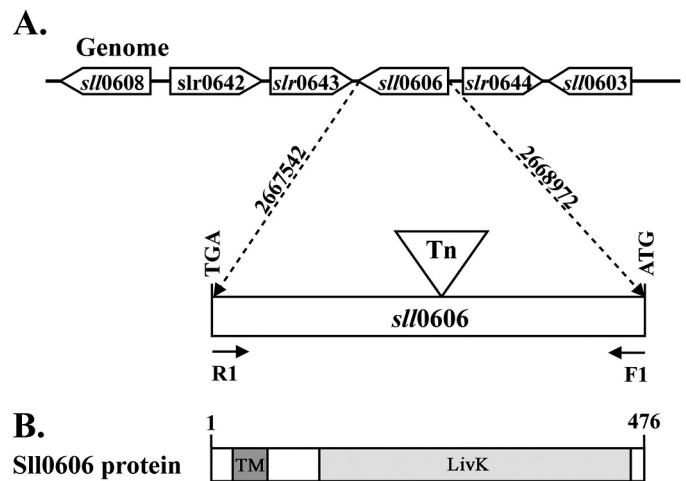


FIGURE 1. Gene organization of *sll0606*, transposon location, and protein features. *A*, gene organization and transposon (*Tn*) location. The *sll0606* gene is located between nucleotides 2667542 and 2668972 of the *Synechocystis* genome and is flanked by the *slr0644* gene encoding a NifR3 homolog and the *slr0643* gene encoding a hypothetical protein. The transposon was inserted between amino acids 220 and 221 of the Sll0606 protein, which caused a truncation of the protein in the *Tsll0606* mutant. *Solid arrows* indicate locations of the two primers R1 and F1 used for complementation analysis. Note that the *sll0603* gene (*MenA*) is located 1143 bp upstream of *sll0606*, and the *sll0608* gene (encoding a hypothetical protein) is located 3542 bp downstream. *B*, features of the Sll0606 protein. The *sll0606* gene codes for a hypothetical protein of 476 amino acids with a single transmembrane domain/transit peptide (TM) from amino acid 19 to 41. The Sll0606 protein contains a branched-chain amino acid-binding domain (*LivK*).

gel strips were excised from the first dimension and equilibrated for 30 min at room temperature with agitation in 50 mM dithioerythritol, 50 mM Tris-HCl, pH 6.8, and 30% glycerol. After equilibration, the gel strips were applied to a LiDS-polyacrylamide resolving gel, and second dimension electrophoresis was performed as described above. Subsequent Western blotting, antibody detection, and blot documentation were performed as described above.

RESULTS AND DISCUSSION

Using inverse PCR, the transposon insertion site in the *Tsll0606* mutant was located. A map for the genes in the vicinity of *Tsll0606* is shown in Fig. 1A. The orientation of the genes that flank *sll0606* (*slr0643* and *slr0644*) indicates that neither of these can form a transcriptional unit with *sll0606*. PCR amplification of the wild type *sll0606* gene from the DNA recipient strain yielded a 1428-bp DNA fragment, which was introduced into the *Tsll0606* mutant background. After this transformation, numerous colonies were identified that were able to grow photoautotrophically in the absence of kanamycin but could not grow in the presence of kanamycin. Several of these were sequenced, and it was confirmed that the wild type *sll0606* gene had been restored in these derivative strains and that the transposon was no longer present (data not shown). This evidence indicates that the transposon interruption of the *sll0606* gene, leading to loss or truncation of the Sll0606 protein, led to the loss of photoautotrophy observed in the *Tsll0606* mutant.

The *sll0606* open reading frame encodes the 476-amino acid Sll0606 protein (Fig. 1B), which contains either a putative trans-

Sll0606 Is Required for PS II Assembly/Stability

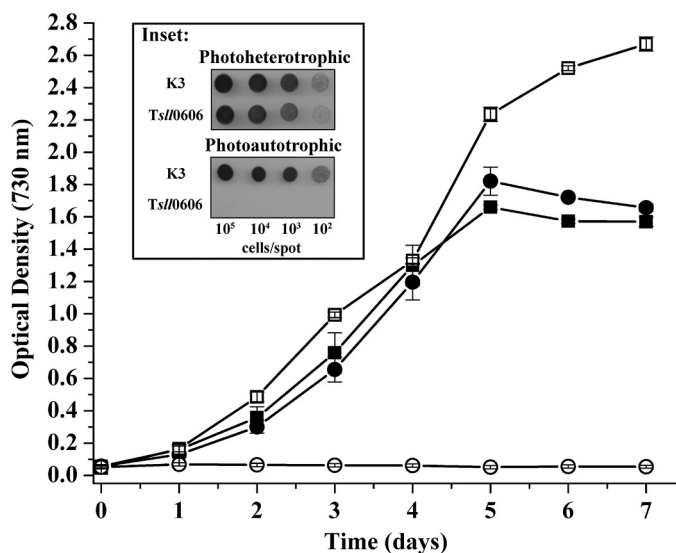


FIGURE 2. Growth of the control strain K3 and Ts//0606 mutant cells in liquid BG-11 medium and on solid BG-11 medium. The optical densities of the photoheterotrophically and photoautotrophically grown cultures were measured daily for 1 week. ■, photoheterotrophically grown K3 cells; ●, photoheterotrophically grown Ts//0606 cells; □, photoautotrophically grown K3 cells; ○, photoautotrophically grown Ts//0606 cells ($n \geq 3$). Error bars, ± 1.0 S.D. In some instances, the S.D. value was smaller than the symbol. Inset, growth of the control strain K3 and the mutant Ts//0606 on solid BG-11 medium. The cells were quantitatively spotted onto the media and grown for 2 weeks before the results were recorded.

membrane domain (32) or a putative *sec* transit peptide (33) near its N terminus. This protein component has been identified in several proteomics experiments (32, 33) as either a plasma membrane protein (32) possessing a single transmembrane helix or as being localized in the periplasm (33). It possesses one recognized domain that is similar to LivK, the branched-chain hydrophobic amino acid transport system substrate-binding protein functioning in an as yet unidentified ABC transporter system. The actual function of Sll0606 is unknown.

To determine the occurrence of the Sll0606 protein in various cyanobacterial lineages, BLAST+ analysis (34) was used to examine the 24 cyanobacterial species considered by Swingley *et al.* (35). Putative orthologs of the Sll0606 protein are found in all fresh water cyanobacteria examined and the diazotrophic marine species. Both *Gloebacter violaceus* and *Acaryochloris marina* contain weaker homologues of Sll0606. Sll0606 is conspicuously absent from all marine, non-diazotrophic species examined (data not shown).

Fig. 2 shows the growth phenotype that results from the integration of a transposon within the *sll0606* gene. In liquid culture under photoheterotrophic conditions, the Ts//0606 mutant grows at the same rate as the control strain. Under photoautotrophic conditions, however, the mutant is unable to grow. Similar results are observed for growth on solid media (Fig. 2, inset). This phenotype appeared quite stable with no apparent accumulation of suppressor mutations that allowed even limited growth under photoautotrophic conditions.

The inability of the mutant to grow photoautotrophically is apparently due to a lack of functional PS II. No oxygen-evolving activity was observed in Ts//0606 cells when using either 2,6-dichlorobenzoquinone or methyl viologen as electron accep-

TABLE 1
Electron transport, fluorescence parameters, and pigment analysis of the control strain K3 and the Ts//0606 mutant

$n \geq 3$, mean ± 1.0 S.D. Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; MV, methyl viologen; DCPIP, 2,6-dichlorophenolindophenol.

Parameter	K3 strain	Ts//0606 mutant
Electron transport ($\mu\text{mol of O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$)		
H ₂ O \rightarrow DCBQ	298 \pm 22	-19 \pm 3
H ₂ O \rightarrow MV	-43 \pm 3	-2 \pm 1
DCPIP + ascorbate \rightarrow MV	-21 \pm 2	-24 \pm 3
Fluorescence		
F_O	0.33 \pm 0.01	1.18 \pm 0.03
F_M	0.64 \pm 0.01	1.22 \pm 0.03
F_V	0.30 \pm 0.01	0.03 \pm 0.01
F_V/F_M	0.48 \pm 0.01	0.03 \pm 0.01
Pigment analysis ($\mu\text{g}/1 \times 10^9$ cells)		
Chl <i>a</i>	5.35 \pm 0.66	5.48 \pm 0.33
Total carotenoids	3.31 \pm 0.24	5.02 \pm 0.31

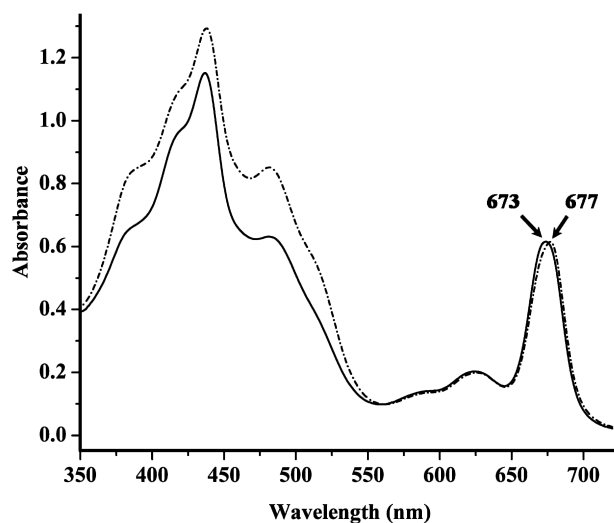


FIGURE 3. Typical visible spectra of 2% dodecyl- β -D-maltoside-solubilized membranes isolated from the control strain K3 and the Ts//0606 mutant. The membranes were isolated as described under "Materials and Methods." The spectra were normalized to the absorbance of the Q_y chlorophyll absorption band. Note the shift in the Q_y absorption maximum in the mutant and the increased absorbance in the 350–550 nm carotenoid/Soret band region. Solid line, control strain K3; dashed line, Ts//0606 mutant.

tors (Table 1). Electron transfer through PS I using 2,6-dichlorophenol indolephenol plus ascorbate as an electron donor to methyl viologen, however, was normal, indicating that the PS I was unaffected in the mutant. Additionally, analysis of the fluorescence parameters shown in Table 1 indicated that Ts//0606 exhibited a large F_O and a small amount of variable fluorescence (F_V) when compared with the control strain. Consequently, the quantum yield for energy trapping by PS II (F_V/F_M) in the mutant was less than 10% of that observed in the control strain.

Fig. 3 illustrates the room temperature absorption spectra of control and mutant dodecyl- β -D-maltopyranoside-solubilized membranes isolated from these strains. The chlorophyll Q_y peak is shifted from 673 nm in the control strain to 677 nm in the mutant. This indicates an enrichment of the longer wavelength-absorbing chlorophylls in the mutant, which are associated with PS I (36). Additionally, in the carotenoid/chlorophyll Soret region (350–500 nm), the overall absorbance has increased relative to the chlorophyll Q_y peak. This spectral difference could indicate an increase in the amount of carotenoids

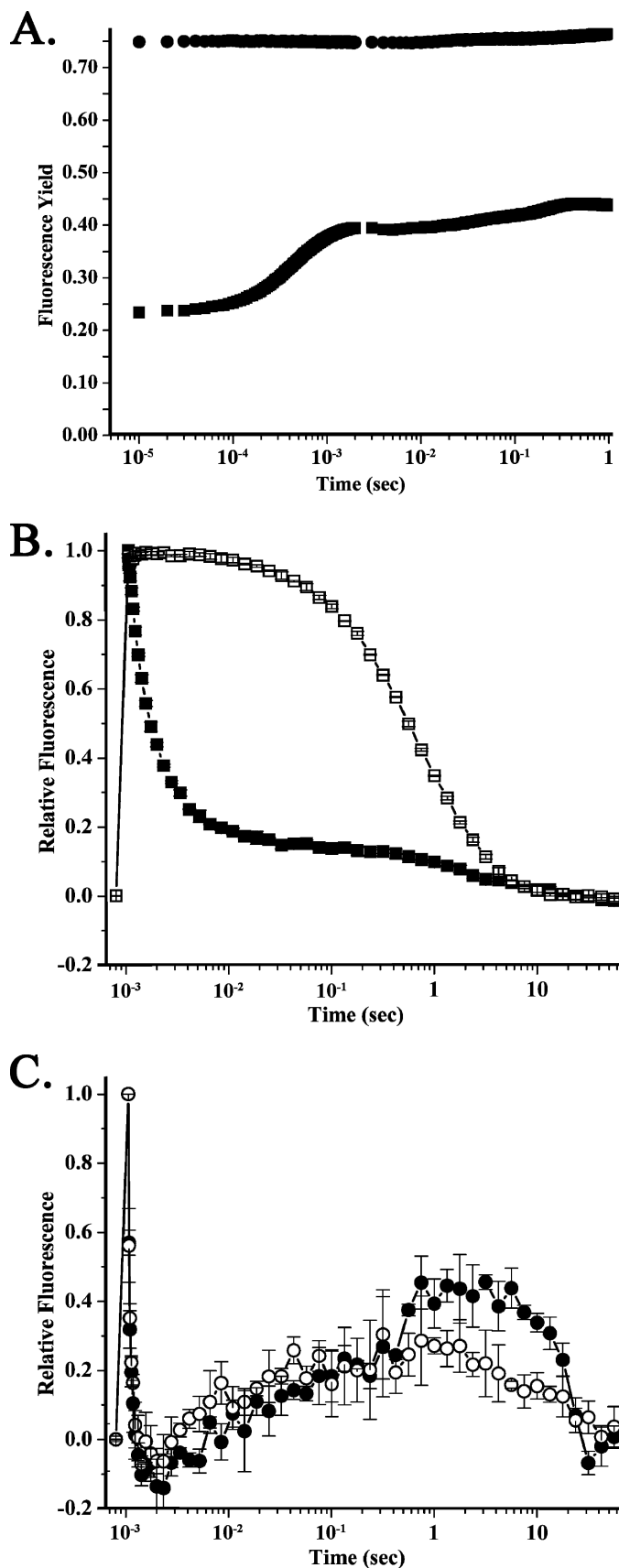


FIGURE 4. Kinetic chlorophyll fluorescence measurements of the control strain K3 and the *Tsll0606* mutant. *A*, typical continuous illumination fluorescence induction experiment conducted on the control strain K3 (■, lower trace) and the *Tsll0606* mutant (●, upper trace). The control strain exhibits a

associated with the membrane, a decrease in the amount of chlorophyll, or both. The first of these possibilities appears to be the case. The amounts of chlorophyll *a* and total carotenoids were determined on a per cell basis and are shown in Table 1. Although chlorophyll *a* is unchanged in the *Tsll0606* mutant, total carotenoids have increased by more than 50%.

Fig. 4 illustrates the fluorescence induction (Fig. 4*A*) and decay (Fig. 4, *B* and *C*) properties of PS II in the control strain and the *Tsll0606* mutant. The fluorescence emission observed under continuous light illumination conditions in the absence of DCMU (Fig. 4*A*) demonstrates that the control strain K3 exhibits the typical O-J-I-P fluorescence transitions associated with the normal stepwise closure of functional PS II reaction centers (37). The mutant, however, exhibits extremely rapid closure with no resolvable transitions. This probably indicates that the bulk of the fluorescence yield observed arises from phycobilisomes or phycobiliproteins that are not connected to functional PS II complexes.

The decay of variable fluorescence either in the absence or presence of DCMU is shown for the control strain and the *Tsll0606* mutant in Figs. 4, *B* and *C*, respectively. A small amount of variable fluorescence was observed in the mutant upon the application of a single saturating flash (note that this variable fluorescence, before normalization, was used to calculate the fluorescence parameters shown in Table 1). For PS II examined in the absence of DCMU, the fluorescence decay is normally dominated by electron transfer from Q_A^- to Q_B , which occurs with a time constant of 200–500 μs. Additional slower decay components are contributed by reaction centers, which must first bind a plastoquinone to the Q_B site before electron transfer occurs, and centers in which charge recombination occurs between Q_A^- and oxidizing-side components (38). The fluorescence decay of the control strain K3 is fully consistent with these earlier observations (Fig. 4*B*, open symbols). The mutant strain exhibited more rapid fluorescence decay (Fig. 4*C*, open symbols). The mutant does not appear to be able to transfer electrons to Q_B ; consequently, the rapid decay of the fluorescence signal in *Tsll0606* may be due to charge recombination with oxidizing-side component(s), possibly Y_Z^* or $P680^+$. This interpretation is supported by the complete loss of oxygen-evolving capability in the mutant (Table 1).

In the presence of DCMU, the PS II reaction centers normally exhibit very slow (seconds) fluorescence decay, which is dominated by charge recombination between Q_A^- and the higher S-states, principally S_2 , of the water-oxidizing complex. The control strain K3 exhibits such decay kinetics (Fig. 4*B*, closed symbols). The fluorescence decay exhibited by the *Tsll0606* mutant in the presence of DCMU (Fig. 4*C*, closed symbols) was very similar to that observed in its absence. This dem-

normal fluorescence induction curve, whereas the mutant exhibits no apparent variable fluorescence under these conditions. *B*, fluorescence decay of the control strain K3 after a single saturating flash both in the absence (■) and presence (□) of 10 μM DCMU. *C*, fluorescence decay of the *Tsll0606* mutant after a single saturating flash both in the absence (●) and presence (○) of 10 μM DCMU. Please note that 1) normalized decay curves are shown in *B* and *C* and that the absolute variable fluorescence of the *Tsll0606* mutant was about 10% of the control strain (Table 1), and 2) the data shown in *B* and *C* are the means of at least three biological replicates (error bars, ±1.0 S.D.). In some instances, the S.D. value was smaller than the symbol.

Sll0606 Is Required for PS II Assembly/Stability

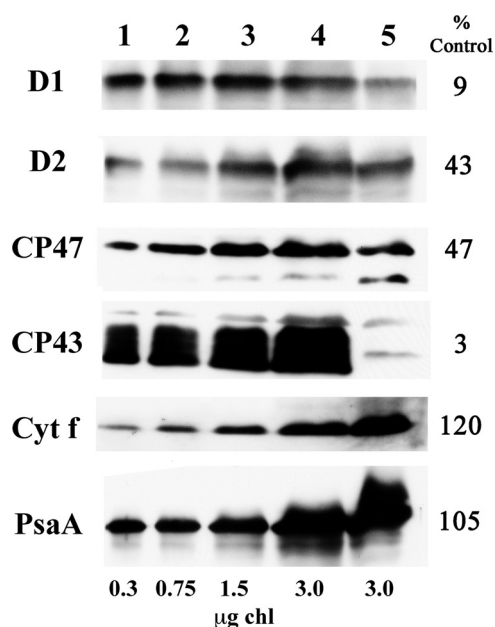


FIGURE 5. Semiquantitative LiDS-PAGE Western blot analysis of the control strain K3 and the *Ts//0606* mutant membrane proteins. The antibodies used as probes are shown on the left. Lanes 1–4 contain varying amounts of control membranes; the chlorophyll load in each lane is shown below and varies from 0.3 to 3.0 μg of chlorophyll/well. Lane 5 contains 3.0 μg of chlorophyll of membranes from the *Ts//0606* mutant. Estimates of the amounts of the various proteins present in the mutant membranes, in comparison with the control strain, are shown to the right.

onstrates that DCMU had no effect on the fluorescence decay kinetics and may indicate that the Q_B site is non-functional. These results indicate that the PS II reaction centers, which do assemble in the *Ts//0606* mutant, are damaged on both oxidizing and reducing sides of the photosystem.

Given the defects apparent in the functional studies of PS II of the mutant, we investigated the protein composition of the photosystem. Fig. 5 illustrates Western blot analysis of membranes isolated from the control strain and the *Ts//0606* mutant, examining a number of PS II components (CP47, CP43, D1, and D2) and two control proteins (PsaA for PS I and cytochrome *f* for the cytochrome b_6-f complex). For this analysis, the LiDS-polyacrylamide gels were loaded on a constant chlorophyll basis. Interestingly, the D1 and CP43 components were dramatically reduced, whereas the CP47 and D2 proteins were modestly affected in the *Ts//0606* mutant. The PsaA protein appeared unaffected in the mutant, whereas significantly more cytochrome *f* accumulated in the mutant than the control strain. These observations are fully consistent with the functional studies described above.

Clearly, the most striking protein defects are the significant losses observed for the CP43, D1, and, to a lesser extent, CP47 and CP43 components. The loss of these proteins would severely compromise PS II function on both the oxidizing and reducing sides of the photosystem because the D1 protein contains the Q_B binding site, and D1 and CP43 provide ligands that coordinate the $Mn_4Ca_1Cl_{1-2}$ cluster that forms the oxygen-evolving site of the photosystem (39, 40).

Two-dimensional blue native/LiDS-PAGE was used to examine the organizational state of several PS II components that accumulate to detectable levels in the *Ts//0606* mutant.

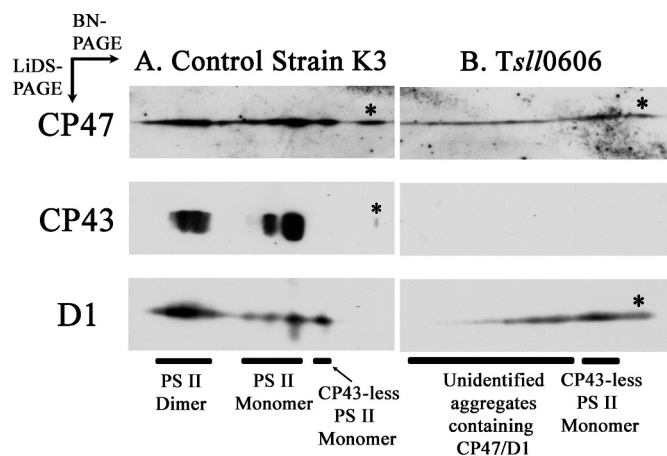


FIGURE 6. Western blot analysis of the control strain K3 and the *Ts//0606* mutant membrane proteins separated by two-dimensional blue native/LiDS-PAGE. A, control strain K3. B, *Ts//0606* mutant strain. The antibodies used as probes are shown on the left. Identification of the PS II complexes resolved in the first dimension by blue native gel electrophoresis is shown below. *, unassembled PS II components.

This technique allows the resolution of the functional complexes associated with the cyanobacterial membranes in the first dimension, whereas the constituent components of these membrane protein complexes are resolved in the second dimension (31). Using this technique, intermediate complexes that participate in PS II assembly and turnover have also been identified (41, 42). In Fig. 6, blue native/LiDS-PAGE was used to resolve the constituent PS II proteins in the control strain K3 and the *Ts//0606* mutant. In the control strain, the PS II dimer, PS II monomer, CP43-less PS II monomer, and unassembled CP47 and possibly a small amount of unassembled CP43 were observed. This pattern was quite similar to those reported previously for *Synechocystis* (31, 41, 43). In the mutant, the CP43-less PS II monomer was observed along with unassembled CP47 and D1. Unidentified aggregates containing CP47 and D1, which were not resolved as discrete complexes in the first dimension, were also consistently observed.

Overall, the functional phenotype of the *Ts//0606* mutant is nearly identical to $\Delta psbC$ mutants lacking CP43 that have been examined previously (44–46). Both mutants exhibit no ability to grow photoautotrophically, do not assemble normal amounts of PS II, and exhibit similar functional defects in the small proportion of PS II reaction centers that do assemble, including loss of the ability to evolve oxygen, loss of DCMU sensitivity, and rapid Q_A^- reoxidation (46). These defects are due to the inability of these mutants to form any fully functional PS II monomers or dimers. These mutants, however, do accumulate the CP43-less PS II monomer (41), which exhibits partial PS II activity. It should be noted that $\Delta psbA$ mutants that contain no D1 protein exhibit no detectable PS II activity. From a protein assembly viewpoint, however, intriguing differences between the $\Delta psbC$ strains and the *Ts//0606* mutant are evident. In the $\Delta psbC$ strains, the D1 protein and CP47 and D2 components are only modestly affected (44) and accumulate to significant levels, being present predominantly as Coomassie-stainable CP43-less PS II monomer (41). $\Delta psbA$ strains, which lack the D1 protein, accumulate CP43, CP47, and D2 in unassembled states (41). In the *Ts//0606* strain, neither D1 nor CP43

accumulates significantly (both <10% of the control; the D1 component that does accumulate assembles into CP43-less PS II monomer), and the CP47 protein is also strongly reduced (~25% of the control). The D2 protein is only modestly affected.

Our results indicate that the Sll0606 protein is required for the accumulation and functional assembly of CP43 and D1 into the PS II reaction center. What is the mechanism of this defect? We cannot answer this question unequivocally at this time. One possible hypothesis is that defective carotenoid transport and/or integration into PS II could be responsible. Recently, it has been shown that the complete suppression of carotenoids biosynthesis had a profound effect on the assembly of PS II (17). Insertional inactivation of the *crtB* and *crtH* genes led to a loss of all detectable carotenoids. Additionally, PS II failed to assemble, whereas the cytochrome *b₆-f* complex was unaffected, and the PS I PsaA component was up-regulated. Most interestingly, the principal PS II protein components affected in this mutant were CP47, CP43, and D1. The accumulation of these components appears to be negatively affected in the absence of a functional carotenoid biosynthetic pathway. This may indicate that the binding of carotenoids is an important determinant for the stability of CP43, CP47, and D1 prior to their integration into a functional PS II complex. Indeed, the overall phenotype of the Δ *crtB/H* double deletion strain was very similar to that which we observe for the *Tsll0606* mutant. The mechanism(s) by which carotenoids are integrated into PS II is unknown at this time. One would predict, however, that the loss of a component required for the transport or assembly of carotenoids into the photosystem would lead to a loss of PS II assembly, with a phenotype very similar to the Δ *crtB/H* mutant, and to an accumulation of carotenoids in the membrane. This is the precise phenotype observed for the *Tsll0606* mutant, which we have described in this paper. One should note that the presence of the LivK domain on Sll0606 indicates the possibility that it binds and participates in the transport of hydrophobic components. One can speculate that in the Sll0606 protein, these sites bind carotenoids.

Alternative hypotheses are of course possible. The Sll0606 protein could act as a chaperone, being required for the stable integration of PS II into the thylakoid membrane. The initial stages in PS II assembly involving D1, D2, and cytochrome *b₅₅₉* appear to take place in the plasma membrane, whereas CP43 and CP47 are found only in the thylakoid compartment (47). The identification of Sll0606 as a plasma membrane/periplasmic protein (32, 33) may suggest a possible link between Sll0606 and the assembly of the D1-D2-cytochrome *b₅₅₉* complex. In this scenario, the loss of the Sll0606 protein would lead to a loss of stability for these components and a failure to assemble functional PS II. The accumulation of carotenoids in the membrane would be a secondary effect, simply a result of the failure of their ultimate destination (*i.e.* PS II) to accumulate. It should be noted that the possible presence of the Sll0606 protein either in the thylakoid membrane or as a luminal component has not been thoroughly investigated at this time. It is possible that the Sll0606 protein is also found in these compartments. In this regard, it should be noted that the PsbO protein is found in both the

periplasm and lumen of the thylakoids (47). Other hypotheses concerning the role of Sll0606 in PS II assembly/stability are, of course, possible. These and other possibilities are currently being investigated.

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Sll0606 Is Required for PS II Assembly/Stability

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