Assembly of Photosystem I

I. INACTIVATION OF THE rbaA GENE ENCODING A MEMBRANE-ASSOCIATED RUBREDOXIN IN THE CYANOBACTERIUM SYNECHOCoccus sp. PCC 7002 CAUSES A LOSS OF PHOTOSYSTEM I ACTIVITY*

Received for publication, February 2, 2002, and in revised form, March 13, 2002 Published, JBC Papers in Press, March 25, 2002, DOI 10.1074/jbc.M201103200

Gaozhong Shen‡, Jindong Zhao, Susan K. Reimer¶, Mikhail L. Antonkine‡, Qun Cai§, Sharon M. Weiland‡, John H. Golbeck‡, and Donald A. Bryant‡**

From the ‡Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802, the †Department of Plant Molecular Biology, College of Life Sciences, Peking University, Beijing, China, and the ¶Department of Biology, St. Francis University, Loretto, Pennsylvania 15940

A 4.4-kb HindIII fragment, encoding an unusual rubredoxin (denoted RubA), a homolog of the Synechocystis sp. PCC 6803 gene slr2034 and Arabidopsis thaliana HCF136, and the psbEFLJ operon, was cloned from the cyanobacterium Synechococcus sp. PCC 7002. Inactivation of the slr2034 homolog produced a mutant with no detectable phenotype and wild-type photosystem (PS) II levels. Inactivation of the rbaA gene of Synechococcus sp. PCC 7002 produced a mutant unable to grow photoautotrophically. RubA and PS I electron transport activity were completely absent in the mutant, although PS II activity was ~80% of the wild-type level. RubA contains a domain of ~50 amino acids with very high similarity to the rubredoxins of anaerobic bacteria and archaea, but it also contains a region of about 50 amino acids that is predicted to form a flexible hinge and a transmembrane α-helix at its C terminus. Overproduction of the water-soluble rubredoxin domain in Escherichia coli led to a product with the absorption and EPR spectra of typical rubredoxins. RubA was present in thylakoid but not plasma membranes of cyanobacteria and in chloroplast thylakoids isolated from spinach and Chlamydomonas reinhardtii. Fractionation studies suggest that RubA might transiently associate with PS I monomers, but no evidence for an association with PS I trimers or PS II was observed. PS I levels were significantly lower than in the wild type (~40%), but trimeric PS I complexes could be isolated from the rbaA mutant. These PS I complexes completely lacked the stromal subunits Psac, Psad, and PsaE but contained all membrane-intrinsic subunits. The three missing proteins could be detected immunologically in whole cells, but their levels were greatly reduced, and degradation products were also detected. Our results indicate that RubA plays a specific role in the biogenesis of PS I.

In oxygen-evolving photosynthetic organisms, two photosystems (PS) cooperate in the utilization of light energy to produce the reducing power and chemical energy required for carbon dioxide fixation. PS II carries out the light-dependent oxidation of water and produces a weak reductant, plastoquinol. PS I catalyzes the light-dependent oxidation of plastocyanin (or cytochrome c55) and produces a strong reductant, reduced ferredoxin (or flavodoxin). The composition and general properties of PS I and II are rather well understood (for reviews, see Refs. 1–3), although important functional details continue to be missing. Using PS I and II complexes purified from the thermophilic cyanobacterium Synechococcus elongatus, structural details for both photosystems have recently been obtained by x-ray crystallography (4, 5).

The PS I reaction center is a large, multisubunit complex that forms trimers in cyanobacterial membranes. The structure of cyanobacterial PS I at 2.5-Å resolution shows that each monomer comprises 12 polypeptides (denoted PsaA–PsaF, PsaI–PsaM, and PsaX), 96 chlorophyll (Chl) a molecules, 22 β-carotene molecules, three [4Fe-4S] clusters, two phylloquinones, and five tightly bound lipids (5). Six redox centers are involved in light-induced electron transfer in PS I (Fig. 1). The PsaA/PsaB heterodimer harbors the primary electron donor P700 (a Chl a dimer), A0 (a monomeric Chl a), A1 (a phylloquinone), and Fx (an interpolyypeptide [4Fe-4S] cluster). Two terminal electron acceptors, Fx and Fd, are [4Fe-4S] clusters that are located on the extrinsic PsaC protein (6). Although significant progress has been achieved in elucidating the structure and function of the PS I, many questions still remain concerning the biogenesis and regulation of PS I in the thylakoid membrane.

The biogenesis of photosynthetic complexes in cyanobacteria and higher plant chloroplasts is a complex multistep process that includes apoprotein translation, protein folding and insertion into the thylakoid membrane, cofactor binding, iron-sulfur cluster assembly, and ordered association of the individual subunits to form multisubunit complexes. It has been demonstrated that all of these steps are highly regulated at the post-translational level, especially for the cofactor- and pigment-binding polypeptides. In recent years, several factors involved in either the assembly or the stability of PS I have been identified (e.g. Refs. 7–9). Nevertheless, the detailed molecular mechanisms of assembly of the photosynthetic apparatus in the thylakoid membranes remain poorly understood. The identification of genes whose products function in the assembly of the
photosystem complexes should greatly increase our understanding of biogenesis and regulation of photosynthesis specifically and protein complexes generally.

The search for proteins that function in the biogenesis of photosynthetic complexes has been greatly facilitated by sequencing of chloroplast genomes, cyanobacterial genomes, the genome of the model plant Arabidopsis thaliana, and the genomes of diverse bacteria. These data, in combination with screens for mutants defective in photosystem assembly in cyanobacteria and diverse eukaryotes, have proven to be significant advantages in the search for such assembly or stabilizing proteins.

In this study, a membrane-associated rubredoxin that is found in cyanobacteria and diverse eukaryotes is described. In contrast to the results of Wastl et al. (13), who concluded that a chloroplast-localized rubredoxin is associated with PS II, it is conclusively demonstrated here that this rubredoxin, denoted RubA, is absolutely required for assembly of functional PS I complexes in cyanobacteria. Mutants lacking RubA produce trimeric PS I complexes, which are inactive in electron transport to flavodoxin or the artificial acceptor methyl viologen. PS I complexes isolated from the rubA mutant contain all membrane-intrinsic subunits but are completely devoid of thestromal PsAC, PsAD, and PsE subunits. A preliminary report of some of these results was presented at the Xth International Congress on Photosynthesis in Budapest, Hungary (14).

MATERIALS AND METHODS

Growth of Cultures—Synechococcus sp. PCC strain 7002 wild-type and mutant strains were grown in medium A supplemented with 1 mg ml\(^{-1}\) NaNO\(_3\) (15). Liquid cultures were grown at 38 °C under constant cool white fluorescent illumination; cultures were bubbled with air supplemented with 1.0% (v/v) CO\(_2\). Agar plates were prepared by solidifying the same growth medium with 1.5% (w/v) Difco Bacto-agar supplemented with 0.3% (w/v) sodium thiosulfate and the following anti-

biotics as appropriate: kanamycin (100 \(\mu\)g ml\(^{-1}\)), erythromycin (25 \(\mu\)g ml\(^{-1}\)), streptomycin (100 \(\mu\)g ml\(^{-1}\)), spectinomycin (50 \(\mu\)g ml\(^{-1}\)), and ampicillin (10 \(\mu\)g ml\(^{-1}\)). For strains lacking PS I activity, the medium was supplemented with 10 mM glycerol as carbon and energy source, and cells were grown at low light intensity (\(-5–10\) \(\mu\)E m\(^{-2}\) s\(^{-1}\)). Growth was monitored turbidimetrically at 730 nm with a Cary-14 spectrophotometer modified for computerized data acquisition by On-Line Instruments, Inc. (Bogart, GA).

Mutant Construction and Complementation—Two open reading frames (ORFs) with strong sequence similarity to ORFs str2033 and str2034 of Synechocystis sp. PCC 6803 are found upstream from the rubEF/FLJ operon of Synechococcus sp. PCC 7002 (see Figs. 2A and 2C). Each gene was inactivated by insertion of a 1.3-kb DNA fragment that encodes the aphII gene and confers resistance to kanamycin. ORF str2033 was inactivated by inserting the fragment at the unique BglII site within the coding sequence, whereas ORF str2034 was inactivated by inserting the resistance cassette into a SmaI site (see Fig. 2). Transformation of these constructions into strains of Synechococcus sp. PCC 7002 was performed as described (15, 16). After selection of kanamycin-resistant transformants and restreaking to allow segregation of alleles, segregation was verified by PCR using appropriate oligonucleotide primers and by Southern blot hybridization analyses.

To verify the phenotype of the ruba::aphII mutant strain, complementation studies were performed with the full-length rubA gene of Synechocystis sp. PCC 7002 and Synechococcus sp. PCC 6803. The complementing genes were introduced into the mutant strain in trans on plasmids pAQE/7002FL and pAQE/6803FL, respectively. To construct plasmid pAQE7002FL, a 360-bp DNA fragment encoding the full-length ruba gene of Synechocystis sp. PCC 7002 was amplified by PCR and cloned into plasmid pET3d. This ruba gene was then excised by digestion with BglII and EcoRI and ligated into plasmid pAQE19 (17), which had also been digested with BglII and EcoRI. Similarly, the full-length ruba gene of Synechocystis sp. PCC 6803 was amplified by PCR and first cloned into plasmid pSE280 (18). A BglII-EcoRI fragment containing the promoter and the ruba gene was recombined into plasmid pAQE19 to produce plasmid pAQE/6803FL. The two complementation plasmids were introduced into the ruba::aphII mutant strain by transformation, and ampicillin- and kanamycin-resistant colonies were selected and characterized.

To determine whether the hydrophobic C-terminal extension of RubA was essential to its function, a 204-bp fragment encoding the N-terminal 68 amino acid residues of the ruba gene and a subsequent stop codon were generated by PCR amplification and cloned into plasmid pSE280 (18). An EcoRI fragment encoding the promotor and the truncated RubA gene was then recloned into an EcoRI site immediately upstream from a spectinomycin-resistance cassette in plasmid pLAT4. Plasmid pLAT4 was generated by cloning a 2.3-kb DNA EcoRI fragment encoding the dispensable desE gene of Synechococcus sp. PCC 7002 (19) into plasmid pBluescript KS (+). A 400-bp HindIII-EcoRI fragment was deleted and replaced with a 2-kb spectinomycin-resistance cassette. The construction pLAT4/6803TR was used to transform the ruba::aphII mutant. Selection of kanamycin- and spectinomycin-resistant mutants produced a strain in which the truncated ruba gene and the promoter were inserted into the Synechococcus sp. PCC 7002 genome at the desE locus.

To test whether the Clostridium pasteurianum rubredoxin could complement the ruba::aphII mutant strain, codons 1–48 of the C. pasteurianum rubredoxin gene (20) were amplified by PCR and fused to residues Glu\(^{50}\) to Asp\(^{115}\) of the Synechocystis sp. PCC 7002 RubA. Due to the introduction of an EcoRI site, residue Glu\(^{50}\) of C. pasteurianum rubredoxin (Glu\(^{50}\) of RubA) was replaced by glutamate. To assemble this construct, an EcoRI-HindIII fragment encoding the C terminus of the RubA protein was cloned in pBluescript. An NdeI-EcoRI fragment encoding residues 1–48 of C. pasteurianum rubredoxin was cloned into pET3d, and subsequently the EcoRI-BamHI fragment encoding the C terminus of RubA was inserted into this plasmid as well. Finally, a BglII-HindIII fragment encoding the complete chimeric gene was cloned into pAQE19 that had been digested with BglII and BamHI. This plasmid was then transformed into the ruba::aphII mutant strain of Synechococcus sp. PCC 7002, and transformants resistant to ampicillin and kanamycin were selected and characterized.

Expression and Purification of the RubA Protein—The full-length ruba gene from Synechococcus sp. PCC 7002 was amplified by PCR using the following primers: 5′-ATACATTCTGAAACATGCATCGCCCATCAACGCAAGATGAGG-3′ and 5′-TCTTTGCTATGGGGATCCTTAGTCCAGACCAT-3′. The 380-bp amplified fragment was digested with BglII and BamHI (sites are underlined in the primer sequences) and ligated into pET3d (Novagen, Madison, WI), which had been digested with NcoI and NotI.
BanHI. The resulting expression plasmid was designated pET3d/7002FL. A similar construction strategy was employed for the expression of a truncated, water-soluble RubA fragment (residues 1–68; see Fig. 3), except that a stop codon was introduced at codon 69 of the gene. These plasmids were introduced into *E. coli* strain BL21(DE3); protein expression occurred after induction with standard protocols.

For the production of antibodies to RubA, a truncated RubA protein that comprised residues 1–94 and lacked the 21-residue C-terminal hydrophobic region, was fused to the C terminus of glutathione S-transferase (GST). The primers used for amplification were 5′-AGCGATCTAGGGCATGCCCATTAG-3′ and 5′-CGCCGGACTTATGTTTCTTTGTTTGGAG-3′. The amplified fragment was digested with BamHI (sites are underlined in the primers) and ligated into the resulting vector. The resulting plasmid was denoted pGST-RubA and was transformed into *E. coli* strain BL21. An overnight culture of this strain was induced with isopropyl-β-D-thiogalactoside at 28 °C for 6 h. Cells were harvested by centrifugation, washed with buffer, and disrupted by two passages through a French pressure cell at 124 megapascals at 4 °C. Thylakoid membranes were diluted to a concentration of 0.4 mg of protein/ml. The thylakoid membranes were resuspended in 50 mM HEPES-NaOH, pH 7.0, buffer containing 0.03% DM prior to further fractionation. For electron transport measurements from H₂O to Q₅, 0.5 mM malonate, and 15% (v/v) glycerol was added as artificial electron acceptors.

**Chlorophyll Analysis and Oxygen Evolution Assay**—Cellular Chl content and whole-chain electron transport activities based on oxygen evolution were calculated on the basis of equal cell numbers determined turbidimetrically by the optical density at 730 nm (OD₇₃₀). These measurements were made with cells harvested by centrifugation from the late exponential growth phase and resuspended in 25 mM HEPES-NaOH, pH 7.0, buffer. Chl was extracted from cells with 100% methanol, and its concentration was determined as described (25) using a Cary-14 spectrophotometer modified for computerized data acquisition by OnLine Instruments, Inc. (Bogart, GA).

Whole-chain oxygen evolution measurements on *Synechococcus* sp. PCC 7002 wild-type and mutant cells were performed using a Clark-type electrode as described (16). The actinic light from a tungsten-halogen lamp was filtered through 3 cm of water and a Corion 500-nm cut-off filter. The saturating light intensity used was about 2300 μE m⁻² s⁻¹. The temperature of the measuring chamber was maintained at 30 °C by a circulating water bath. Cell samples were adjusted to a final concentration of 5 μg of Chl ml⁻¹ or 1.0 OD₇₃₀ ml⁻¹ in 25 mM HEPES-NaOH, pH 7.0, buffer. For whole-chain electron transport measurements, H₂O to CO₂, 5 μM NaHCO₃ was added to the cell suspension. For electron transport measurements from H₂O to Q₅, 0.5 mM KFe(CN)₆ and 0.1 mM 2,5-dimethyl-p-benzazone were added as artificial electron acceptors.

**Fluorescence Emission Spectra at 77 K**—Fluorescence emission spectra at 77 K were measured using a SLM 8000C spectrophotometer as described (23). The excitation wavelength was 730 nm; the emission wavelength was 800 nm. The temperature of the measuring chamber was maintained at 30 °C by a circulating water bath. Whole-cell samples were adjusted to a final concentration of 5 μg of Chl ml⁻¹ or 1.0 OD₇₃₀ ml⁻¹ in 25 mM HEPES-NaOH, pH 7.0, buffer. Glycerol was added to a final concentration 60% (v/v). Cells were frozen at a concentration of 1.0 OD₇₃₀ ml⁻¹. The excitation wavelength was 440 nm for Chl excitation. A long pass filter (transmitting at >600 nm) was used at the inlet of the emission monochromator to minimize contributions from scattered light.

**PS I-mediated Electron Transport Rates**—PS I-specific electron transport rates were measured for thylakoid membranes isolated from *Synechococcus* sp. PCC 7002 strains by measuring the rate of oxygen uptake via the Mehler reaction. Photosynthetic electron transport from 2,6-dichlorophenoldiphenol to methyl viologen (MV) catalyzed by PS I was monitored by measuring the oxygen uptake under red light illumination (16). The actinic light from a tungsten-halogen lamp was filtered through 3 cm of water and a Corion 600-nm cut-off filter was used for excitation light. Thylakoid membranes were resuspended in the 50 mM HEPES-NaOH, pH 8.0, buffer, containing 5 mM MgCl₂, 10 mM CaCl₂, 0.5% (v/v) dimethyl sulfoxide, and 15% (v/v) glycerol for storage. PS I complexes were resuspended in 50 mM Tris-HCl, pH 8.0, buffer at a Chl concentration of 5 μg of Chl ml⁻¹, 2,6-Dichlorophenoldiphenol (0.1 mM) and sodium ascorbate (1 mM) were added as electron donors, and MV (1 mM) was added to the reaction mixture as the electron acceptor. The reaction mixture also contained 20 mM 3-(3,4-dichlorophenyl)-1,1-dimethyleurea and 5 mM NaN₃.

The electron transport activity of isolated PS I complexes was measured by determining the rate of flavodoxin reduction as described previously (26–28). PS I complexes were suspended in 50 mM Tris-HCl, pH 8.0, buffer at a Chl concentration of 5 μg ml⁻¹. The reaction mixture
As shown in Fig. 3, the deduced amino acid sequence of the slr2033 homolog contains a 50-amino-acid domain with very high sequence similarity to rubredoxins from numerous organisms. Rubredoxins are small non-heme iron proteins, which are widely distributed among eubacteria and archaea, especially anaerobes (31). The product of the Synechocystis sp. PCC 7002 slr2033 homolog has a calculated molecular mass of 12.5 kDa and exhibits highest sequence similarity to the rubredoxin-like proteins encoded by ORF slr2033 of Synechocystis sp. PCC 6803 (32) and slr3843 of Nostoc sp. PCC 7120 (33). Compared with bacterial rubredoxins, the cyanobacterial rubredoxins have a charged, hydrophilic N-terminal extension of ~14 amino acid residues as well as a longer C-terminal extension of ~50 residues. This extension is predicted to form a hydrophobic, transmembrane α-helix at its end and is connected to the rubredoxin domain by a region predicted to form a flexible hinge (see Fig. 3). Based upon this sequence similarity to bacterial rubredoxins and upon the results from the characterization of the protein as presented below, we have assigned the slr2033 homolog of Synechococcus sp. PCC 7002 the gene locus designation rubA. Genes encoding proteins similar to rubA have also been identified in the nucleomorph genome of the eukaryotic cryptomonad Guillardia theta (34) and the nuclear genome of the higher plant A. thaliana (35) (see Fig. 3).

Overexpression of the rubA Gene and Characterization and Distribution of RubA—Attempts to overproduce the full-length RubA protein were not successful, but overproduction of a truncated form of the protein in which a stop codon was inserted at codon 69 was achieved. The resulting protein was partly purified by ion exchange chromatography, and the absorption spectrum of this protein is shown in Fig. 4. The spectrum contains characteristic absorption features of sulfur → iron charge-transfer transitions; is characterized by absorption maxima at 375, 475, and 570 nm; and is very similar to the spectra of typical bacterial rubredoxins (20, 36, 37). The EPR spectrum of oxidized RubA is also shown in Fig. 4. This spectrum features the characteristic signals from a ground S = ½ spin state near the rhombic limit (E/D) of ¼. These spectra are characterized by a nearly isotropic resonance around g = 4.31 derived from the middle Kramers doublet and a resonance at g = 9.46 that represents the lowfield peak of a rhombic signal. The predicted g = 0.86 derivative and g = 0.61 trough are broadened, are not easily detected, and indeed were not observed here. These spectral features are identical to those observed for the rubredoxin of the anaerobic bacterium C. pasteurianum (38) and are similar to the soluble fragment derived from the nucleomorph-encoded rubredoxin of the cryptomonad alga G. theta (13, 39). These data indicate that the water-soluble, rubredoxin-like domain of cyanobacterial RubA...
binds a non-heme iron atom and thus represents a typical rubredoxin.

For the production of antibodies, the water-soluble N-terminal domain of RubA was fused to GST. The overproduced protein was purified on a GST affinity column, digested with thrombin, and repurified by gel filtration. Fractions containing rubredoxin were pooled, concentrated, and lyophilized, and the protein was judged to be homogeneous by SDS-PAGE analysis. N-terminal amino acid sequencing was performed to verify the correctness of the purified protein, which was used to raise antibodies in rabbits.

Antibodies to the soluble, recombinant RubA protein were used to examine the subcellular localization of RubA. Antibodies to the RubA protein cross-reacted with a protein with an apparent mass of 13 kDa in total membranes isolated from Synechococcus sp. PCC 7002 (Fig. 5, B and C, lane 1) and from Synechocystis sp. PCC 6803 (Fig. 5A, lane 1). These results indicate that RubA is probably a membrane-intrinsic protein, as suggested by the presence of the predicted transmembrane α-helix at the C terminus. An immunoreactive protein with an apparent mass of about 8 kDa (see Fig. 5) was usually detected as well and probably represents a degradation product, since the amount of this species increased in samples that had been stored for increasing times. Interestingly, the antibodies also cross-reacted strongly with proteins of 13 and 8 kDa in thylakoid membranes isolated from C. reinhardtii (Fig. 5C, lane 2) and spinach (Fig. 5C, lane 3). Using very highly purified plasma (Fig. 5A, lane 2) and thylakoid (Fig. 5A, lane 3) membranes from Synechocystis sp. PCC 6803 (40), it can be seen that RubA is only found in the thylakoid membrane fraction. Similar results were obtained with Synechococcus sp. PCC 7002 membrane fractions prepared by the same method (data not shown). The antibodies did not cross-react with proteins in highly purified PS I trimers prepared with Triton X-100 (Fig. 5B, lane 2) or DM (Fig. 5B, lane 3) but cross-reacted strongly with a fraction enriched in PS I monomers prepared with the latter detergent (Fig. 5B, lane 4).

Since a previous study had suggested that the rubredoxin might be associated with PS II in spinach (13), the PS I monomer fraction, which typically contains some contaminating PS II complexes, was subjected to further purification by ion exchange chromatography. A fraction from a sucrose gradient containing a mixture of PS I monomers, PS II, and other proteins was applied to a DEAE-Sephacel column, extensively washed with starting buffer, and developed with a linear sodium chloride gradient (0–400 mM). Fractions were collected and subjected to SDS-PAGE, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane for immunoblotting analysis. As shown in Fig. 6, RubA was detected in fractions 2–6, and the fraction containing the greatest amount of the RubA protein was fraction 3. The distribution of PS I monomers was evaluated by simultaneously determining the elution pattern of the PS I-specific protein PsaD. PsaD was detected in fractions 2–6, with the greatest amount of this

**Fig. 3.** Amino acid sequence comparison of rubredoxin-like proteins from cyanobacteria with other rubredoxin sequences. An alignment is shown of amino acid sequences of rubredoxins from Synechococcus sp. PCC 7002 (this work) (1); Synechocystis sp. PCC 6803 (32) (2); Anabaena sp. PCC 7119 (3); Nostoc sp. PCC 7120 (33) (4); G. theta (34) (5); A. thaliana (35) (6); C. tepidum rubredoxins 1, 2, and 3 (56) (7–9); and C. pasteurianum (20) (10). The total number of amino acid residues for each polypeptide is shown at the end of each sequence. The domains (rubredoxin, hinge, and membrane anchor transmembrane α-helix) are indicated above the sequences, and the four iron-binding cysteine residues are indicated in boldface type. *Hyphens* indicate insertions/deletions to optimize the sequence similarity.
protein occurring in fraction 3. The distribution of PsaD thus appears to be nearly identical to the distribution of RubA. However, the observed distribution of RubA and PsaD differs from that for CP43 (PsbC), a subunit of PS II, which was detected in fractions 4–7 and which was maximal in fraction 4 (see Fig. 6). These results demonstrate that the RubA protein copurifies with the PS I monomer fraction but not with PS II complexes. This result is consistent with, but does not necessarily prove, the hypothesis that RubA can bind to PS I monomers.

**Mutagenesis of the rubA Gene**—The rubA gene was inactivated by inserting a 1.3-kb *BamHI* DNA fragment that encodes the *aphH* gene and confers kanamycin resistance into the unique *BglII* site within the *rubA* coding sequence (Fig. 2B). This construction was used to transform three *Synechococcus* sp. PCC 7002 strains: the wild type, a PS I-less strain created by complete deletion of the *psaA* and *psaB* genes (16), and a PS II-less strain created by inactivation of the *psbCD* and *psbB* genes.2 Transformants carrying the insertionally inactivated

---

2 G. Shen and D. A. Bryant, unpublished results.
rubA::aphII gene were easily and fully segregated in the PS I-less ΔpsaAB strain. Full segregation of the rubA::aphII and rubA alleles in the ΔpsaAB strain was verified both by Southern blot hybridization analyses (data not shown) and by PCR (Fig. 2C). As shown in Fig. 2C, PCR amplification with DNA from Synechococcus sp. PCC 7002 wild-type cells using primers flanking the rubA gene amplified a 330-bp DNA fragment. PCR amplification with DNA from the rubA mutant strain using the same primers amplified a 1.6-kb DNA fragment as expected (see Fig. 2, B and C).

The rapid segregation of the rubA and rubA::aphII alleles in the ΔpsaAB background suggested that RubA might play a role in PS I biogenesis. To test this possibility, the ΔpsaAB and ΔpsaAB rubA::aphII strains were complemented at the psaAB locus by transformation with plasmid pAQEEmr80. This plasmid contains the wild-type psaAB genes (41) with the erm gene, that conveys resistance to erythromycin, inserted at the EcoNI site that occurs 112 bp downstream from the stop codon of the psaB gene. The resulting pseudo-wild-type strain, produced by complementation from the PS I-less ΔpsaAB strain, is referred to here as strain WTREM. This strain has a phenotype that is identical to that of the true wild-type strain (strain PR6000) of Synechococcus sp. PCC 7002 as indicated by its ability to grow photoautotrophically at wild-type rates, by its Chl content, and by its ability to assemble functional PS I reaction centers (see Table I, and see below). Because complementation of the ΔpsaAB strain results in a wild-type phenotype, complementation of the psaAB locus in the ΔpsaAB rubA::aphII strain should produce a strain that is equivalent to inactivation of the rubA gene in a wild-type genetic background.

Thylakoid membranes of the pseudo-wild-type WTREM and rubA::aphII strain were subjected to immunoblotting analysis with antibodies to RubA. As shown in Fig. 7, lane 1, thylakoids of strain WTREM exhibit a strong cross-reaction with the an-
Assembby of Photosystem I

**Table I**

|                       | WT<sup>a</sup> | WTREm<sup>b</sup> | rubA mutant<sup>c</sup> |
|-----------------------|---------------|------------------|------------------------|
| **Photoautotrophic growth (h)** |               |                  |                        |
| At 5–10 μE m<sup>–2</sup> s<sup>–1</sup> | 62 ± 2.7 | 64 ± 3.4 | No growth |
| At 250 μE m<sup>–2</sup> s<sup>–1</sup> | 4.4 ± 0.3 | 4.2 ± 0.4 | No growth |
| **Photomixotrophic growth (h)** |               |                  |                        |
| At 5–10 μE m<sup>–2</sup> s<sup>–1</sup> | 17 ± 1.5 | 16 ± 1.8 | 34 ± 2.1 |
| At 250 μE m<sup>–2</sup> s<sup>–1</sup> | 3.4 ± 0.4 | 3.3 ± 0.3 | No growth |
| Chlorophyll content (mg of Chl OD<sub>730</sub>–1) | 4.46 ± 0.28 | 4.57 ± 0.24 | 2.49 ± 0.21 |
| **Oxygen evolution (μmol O<sub>2</sub> OD<sub>750</sub>–1 s<sup>–1</sup>)** | 1330 ± 60 | 1280 ± 45 | NM<sup>d</sup> |
| Whole chain (H<sub>2</sub>O to CO<sub>2</sub>) | 1960 ± 130 | 1940 ± 120 | 1490 ± 160 |
| PS II-mediated electron transport<sup>e</sup> | 510 ± 40 | 530 ± 57 | NM |
| DCPIP to MV (μmol O<sub>2</sub> mg of Chl<sup>–1</sup> h<sup>–1</sup>) | 6710 ± 340 | 6650 ± 390 | NM |

<sup>a</sup> Wild type.  
<sup>b</sup> ΔpsaAB mutant strain complemented with psaAB genes.  
<sup>c</sup> rubA::aphII.  
<sup>d</sup> Chlorophyll was extracted from cells were grown photomixotrophically at light intensity of 5–10 μE m<sup>–2</sup> s<sup>–1</sup>.  
<sup>e</sup> Measurements were made on whole cells grown at the exponential phase.  
<sup>f</sup> NM, not measurable.  
<sup>g</sup> Measurements were made on thylakoid membranes.

Fig. 7. Immunoblot analysis with antibodies against the Synechococcus sp. PCC 7002 RubA protein. Lane 1, thylakoids of Synechococcus sp. PCC 7002 strain WTREm; lane 2, thylakoids of the rubA mutant; lane 3, thylakoids of the rubA mutant complemented with a plasmid encoding the full-length rubA gene of Synechocystis sp. PCC 6803; lane 4, whole-cell extracts of the rubA mutant complemented with a plasmid encoding a truncated, soluble fragment of Synechocystis sp. PCC 6803 RubA; lane 5, fraction eluted at 0.2 M NaCl from DEAE-Sephacel chromatography of the supernatant containing soluble proteins from the rubA mutant complemented with a plasmid encoding a water-soluble fragment of Synechocystis sp. PCC 6803 RubA. Molecular mass markers (kDa) are indicated on the right. The arrow point to the RubA protein and truncated, soluble RubA domain (RubA<sub>TR</sub>). 40 μg of protein was loaded per lane.

tobodies to RubA, but no cross-reaction was observed for thylakoids from the rubA::aphII mutant. Thus, the rubA::aphII mutant lacks any detectable RubA as expected.

**Photoautotrophic Competence and Complementation of the rubA Mutant**—Growth of the WTREm strain was indistinguishable from that of the true wild-type strain under photoautotrophic conditions (Table I). However, the rubA::aphII strain was completely unable to grow photoautotrophically (Table I). When the medium was supplemented with 10 mM glycerol as the carbon source, this strain was able to grow photomixotrophically at very low light intensity (5–10 μE m<sup>–2</sup> s<sup>–1</sup>), but the rubA mutant was unable to tolerate low light intensities greater than about 50 μE m<sup>–2</sup> s<sup>–1</sup>. As shown in Table I, the rubA::aphII mutant had a doubling time of 34 h under low light, photomixotrophic conditions; this is significantly longer than that for the wild-type strain under the same conditions (17 h). These results indicate that RubA plays a role in establishing the normal photosynthetic electron transport activities of cyanobacterial cells and that the rubA mutant cells display sensitivity to high light intensities, a phenotype often observed with mutants deficient in PS I (42).

**Complementation of the rubA Mutant**—To verify the phenotype of the rubA::aphII mutant, the mutant was complemented by transforming the strain with plasmid pAQE7002FL, which encodes the wild-type rubA gene of Synechococcus sp. PCC 7002. The complemented mutant strain grew photoautotrophically at a rate equivalent to that of the wild-type strain (data not shown). Complementation of the rubA::aphII mutant was also performed using plasmid pAQE19/6803FL, which encodes the wild-type rubA gene of Synechocystis sp. PCC 6803. This strain also exhibited a growth rate under photoautotrophic conditions that was equal to that of the pseudo-wild-type strain WTREm (data not shown). Immunoblotting analysis (Fig. 7, lane 3) showed that the amount of RubA protein in this complemented strain was similar to that for the pseudo-wild-type strain WTREm (Fig. 7, lane 1). These results establish clearly that the growth defect observed in the rubA::aphII mutant is only due to the rubA mutation and rule out any possibility of a defect caused by a secondary mutation.

The primary difference between the cyanobacterial rubredoxin, RubA, and other bacterial rubredoxins is the additional 50 amino acids at the C terminus and in particular the predicted transmembrane α-helix that occurs near the C terminus of the protein. To evaluate the role of this domain of RubA, the rubA::aphII strain was transformed with plasmid pLAT4/6803TR, which carries the Synechocystis sp. PCC 6803 rubA gene, which has been modified by site-directed mutagenesis to include a stop codon at residue 69 (see Fig. 3). Selection of spectinomycin- and kanamycin-resistant transformants resulted in a strain that can only produce the water-soluble, N-terminal rubredoxin-like domain of RubA. This strain was unable to grow photomixotrophically (data not shown), although the water-soluble, rubredoxin-like domain was detected in cell extracts by immunoblotting analysis (Fig. 7, lane 5). These results suggest that the hydrophobic C-terminal region of the RubA protein might have an important and specific function.

One additional protein was tested for its ability to complement the rubA::aphII mutation. A chimeric rubredoxin gene was constructed from codons 1–48 of the rubredoxin gene of *C. pasteurianum* (a kind gift from Dr. Donald Kurtz, University of Georgia), and codons 63–115 of the rubA gene of *Synechococcus* sp. PCC 7002. To facilitate the gene construction, residues 48 of *C. pasteurianum* rubredoxin was changed from glu-
tamine to glutamate, and the six C-terminal residues of this rubredoxin were replaced by residues 63–115 (hinge and transmembrane α-helix) of RubA of *Synechococcus* sp. PCC 7002. When the *rubA*:aphII strain was complemented with this chimeric rubredoxin gene, the resulting strain could not grow photoautotrophically (data not shown). This result suggests that the C-terminal modified *C. pasteurianum* rubredoxin does not have the structural and/or functional properties necessary to replace the RubA protein of cyanobacteria. It is possible that the inability to complement the *rubA* mutant strain is due to the absence of the N-terminal extension that is found in the cyanobacterial RubA proteins (see Fig. 3) but that is missing in bacterial rubredoxins.

**Chlorophyll Content and 77 K Fluorescence Emission of the *rubA* Mutant**—Table I shows a comparison of the Chl contents of three strains: the wild type, the pseudo-wild-type WTREm, and the *rubA*:aphII mutant. The *rubA* mutant cells contained about half of the Chl of the control cells. The carotenoid content of the mutant cells was slightly higher than in the control cells, but phycobiliprotein levels were essentially identical in all three strains (data not shown).

Most of the Chl in cyanobacteria is associated with the two photosynthetic complexes, PS I and II (16). Thus, fluorescence emission at low temperature is a convenient way to detect differences in the PS I/PS II ratio in thylakoid membranes. Fig. 8 shows the fluorescence emission spectra at 77 K of equivalent numbers of cells of the *rubA* mutant and pseudo-wild-type WTREm strains. For the WTREm strain, excitation of whole cells at 440 nm resulted in a typical, large emission peak at 716–717 nm, which results from the excitation of PS I-associated fluorescence emission at 717 nm. In contrast, PS II-mediated oxygen evolution was detected for the *rubA* mutant, although a substantial PS II activity (77% of wild-type level) was measured when PS II-mediated oxygen evolution activity was assayed with ferricyanide and 2,5-dimethyl benzoquinone as electron acceptors. These results indicate that the photosynthesis defect in the *rubA* mutant is mostly likely due to a defect in PS I.

To explore this possibility further, the oxygen uptake rate for PS I-mediated electron transport from 2,6-dichlorophenolindophenol to methyl viologen (Mehler reaction) was measured for the WTREm and *rubA* mutant strains. As shown in Table I, the oxygen uptake rate (530 μmol of O₂ mg of Chl⁻¹ h⁻¹) for thylakoids from the pseudo-wild-type WTREm was essentially identical to that of the wild type strain, but thylakoids from the *rubA* mutant had no detectable PS I-mediated oxygen uptake. PS I activity was also measured by determining the rate of electron transfer from the physiological donor, cytochrome c₅₅₃ to a physiologically relevant acceptor, flavodoxin. As shown in Table I, thylakoids from the WTREm strain had a PS I-mediated rate of flavodoxin reduction (6650 μmol mg of Chl⁻¹ h⁻¹) similar to that of wild-type thylakoids. However, consistent with the results from the Mehler reaction studies, thylakoids of the *rubA* mutant exhibited no measurable flavodoxin reduction. These results clearly establish that the PS I reaction centers of the *rubA* mutant strain are not functional in electron transport.

**FIG. 8.** 77 K fluorescence emission spectra. Fluorescence emission spectra of the *Synechococcus* sp. PCC 7002 WTREm strain (solid line) and the *rubA* mutant (dotted line). Each spectrum is the average of five independent measurements. The excitation wavelength was 440 nm.
Assembly of Photosystem I

Assembly of the PS I Complex—The rate of oxygen evolution measured for whole-chain electron transport and the absence of detectable rates of PS I-mediated electron transport demonstrated that the rubA mutant lacks functional PS I reaction centers. However, 77 K fluorescence emission measurements indicated that the mutant produced a reduced but nevertheless substantial amount of PS I. To investigate the effect of the rubA mutation on PS I composition and assembly, thylakoids of the WTREm and rubA mutant strains were subjected to a standard PS I isolation procedure. More than 90% of PS I complexes isolated from the pseudo-wild-type WTREm strain were isolated as trimers. In contrast, trimeric PS I complexes accounted for only 30–35% of the total PS I complexes that could be isolated from the thylakoids of the rubA mutant; the remaining PS I complexes were recovered as monomers. This result suggested that the rubA mutation did not prevent the formation of trimeric PS I complexes but led to a decreased stability of the PS I trimers in thylakoid membranes.

The composition of the PS I complexes isolated from the WTREm and rubA mutant strains was analyzed by SDS-PAGE. As shown in Fig. 9A, no difference was observed in the composition of the integral membrane subunits of the trimeric PS I complexes from the rubA mutant and WTREm strains. PS I trimers of the rubA mutant contained similar amounts of PsaA-PsaB, PsaF, PsaL, PsaK, and the low molecular mass polypeptides PsaI, PsaJ, and PsaM as trimers isolated from control WTREm strain. However, the three stromal subunits (PsaC, PsaD, and PsaE) were completely absent in the trimeric PS I complexes isolated from the rubA mutant.

Immunoblotting analyses of isolated PS I complexes, thylakoids, and whole-cell extracts from the WTREm and rubA mutant strains were performed to verify the absence of PsaC, PsaD, and PsaE in the RubA-less mutant. PsaC, PsaD, and PsaE were undetectable in purified trimeric complexes from the rubA− strain (data not shown). Fig. 9B shows the results of immunoblotting analyses of whole-cell extracts. These data show that the three stromal polypeptides of PS I were detectable in the rubA mutant, but their levels were much reduced relative to those of the control strain. Similarly, low levels of PsaC, PsaD, and PsaE could be detected in the thylakoids of the rubA mutant (data not shown). Faster migrating, immunoreactive species were observed for both PsaC and PsaE (Fig. 9B, arrows). These data suggest that these proteins are unstable and are rapidly degraded in the rubA mutant cells. From these results, it is concluded that PsaC, PsaD, and PsaE are probably synthesized normally in the rubA mutant, but these proteins are more easily lost from PS I complexes during purification and are much less stable than in the wild type.

DISCUSSION

Rubredoxins are typically very small (~6 kDa), water-soluble, mononuclear non-heme iron proteins that occur most frequently in anaerobic eubacteria and archaea. However, rubredoxins have also been found in a few aerobic bacteria (e.g. Pseudomonas oleovorans (46) and Acinetobacter sp. (47)) and more recently have even been found in oxygen-evolving prokaryotes and eukaryotes (see below). Rubredoxins have been spectroscopically, biophysically, and structurally well characterized for many years. The roles of these proteins in cellular metabolism and physiology remain poorly understood, although they have recently been implicated as electron donors or acceptors in diverse biochemical reactions. Reduced rubredoxins have been proposed or shown to serve as electron donors to nitrate reductase (48), hydrogenase (49), rubredoxin:oxygen reductase (50), alkane hydroxylase (47), ruberythrin (51), superoxide reductases (51, 52), and bacterioferritin (53). Several bacterial genomes have been shown to encode at least two rubredoxins, and it is possible that sequence variations in the vicinity of the iron-binding ligands could confer specific functional differences to the different proteins (47, 54–56). The physiological role of electrons to rubredoxin for the above reactions is generally not known, with the exception of those organisms that have been shown to synthesize an NADH:rubredoxin oxidoreductase (see Ref. 37). Interestingly, in the photosynthetic green sulfur bacterium Chlorobium tepidum, whose genome encodes three rubredoxins (Ref. 56; see Fig. 3), a rubredoxin serves as the electron acceptor when pyruvate is oxidized to acetyl-CoA and CO₂ by pyruvate:ferrodoxin oxidoreductase (57).

The complete genome sequence analysis of the cyanobacterium Synechocystis sp. PCC 6803 showed that this organism has the potential to synthesize an unusual rubredoxin-like protein of 115 amino acids (Ref. 32; see Fig. 3). Similar proteins have now been identified in diverse oxygen-evolving prokaryotes including Synechococcus sp. PCC 7002 (this work), Anabaena variabilis strain PCC 7937, Nostoc sp. PCC 7120 (33), Nostoc punctiforme,³ Prochlorococcus marinus strains MED4 and MIT9313,³ and Synechococcus sp. WH8102.³ Rubredoxins with strong sequence similarity to the cyanobacterial rubredoxins were also recently discovered on chromosome 2 of the nucleomorph of the cryptomonad G. theta (34) and on chromosome 1 of the higher plant A. thaliana (35). This broad distribution of RubA homologs among cyanobacteria, prochlorophytes, cryptomonads (red algae), green algae, and higher plants strongly suggests that rubA homologs might be found in all oxygen-evolving photosynthetic organisms.

The nucleomorph represents the remnant nucleus of the red-algal endosymbiont acquired by the eukaryotic ancestor of the cryptomonad. The product of the nucleomorph-encoded rubredoxin gene includes a long leader sequence that is pre-
sumed to direct the protein into the cryptomonad chloroplast. A water-soluble, recombinant form of the \textit{G. theta} rubredoxin has the optical and EPR spectroscopic characteristics of typical rubredoxins (13, 39), and the structure of the water-soluble zinc-rubredoxin domain of the protein has been solved by NMR methods (59). This soluble domain of the recombinant rubredoxin is quite similar in structure to typical bacterial rubredoxins. The full-length rubredoxin of \textit{G. theta} could be imported into pea chloroplasts (13), and by using antibodies to the \textit{G. theta} rubredoxin in immunoelectron microscopy studies, the protein was shown to be localized to thylakoids in \textit{G. theta} and pea chloroplasts. Although rubredoxin was also detected in thin sections of the cyanobacterium \textit{Synechocystis} sp. PCC 6803, labeling of both the thylakoids and the cytoplasm was consistent with the thylakoids of the green alga \textit{C. reinhardtii}.

The presence of an immunologically related protein in spinach thylakoids was confirmed here with an antiserum to a cyanobacterial RubA, and these antibodies also detected a protein of similar mass in thylakoid membranes of the green alga \textit{C. reinhardtii}. RubA was shown to be present in purified thylakoid but not plasma membranes of \textit{Synechocystis} sp. PCC 6803, and the important role of the putative transmembrane \(\alpha\)-helix at the C terminus of the protein was established by complementation studies using a truncated form of the protein.

In typical rubredoxins, three \(\beta\)-strands are joined by two loops, each of which contains two cysteine residues (Cys-X-X-Cys) ligated to an iron atom to form a structure known as a “knuckle” (59). All structurally characterized rubredoxins and rubredoxin-like domains of more complex proteins share significant amino acid sequence identity (~50%; see Fig. 3) and have essentially isostructural \([\text{Fe(S-Cys)}_4]\) sites with distorted tetrahedral coordination geometry (48, 59). Surprisingly, however, the redox potentials of rubredoxins and rubredoxin-like domains vary over a considerable range from about -150 mV to about +230 mV (53). Although the redox potential of the RubA protein was not determined in this study, the soluble domain of the \textit{G. theta} protein was shown to have a midpoint potential of +125 mV (13). The C-terminal extension found in RubA homologs from cyanobacteria and other oxygen-evolving organisms is also highly conserved. This suggests that the flexible hinge region and the membrane anchor are likely to be quite important in the function of the RubA protein. The N-terminal extensions of the cyanobacterial and the eukaryotic RubA homologs are somewhat variable in sequence, but all of these sequences are rather hydrophilic and highly charged. \textit{C. pasteurianum} rubredoxin (midpoint potential ~-55 mV (38)) was unable to complement the rubA mutation, even when fused to the C-terminal extension of a cyanobacterial RubA. This could indicate that the redox potential of this protein is inappropriate to replace RubA function, that the N-terminal extension is very important in the functionality of RubA, that highly specific protein-protein interactions are required for RubA function, or that insufficient expression of the fusion protein was achieved to effect complementation. Further studies will be required to determine which of these possibilities is correct.

The results presented here clearly establish that the loss of the RubA rubredoxin causes the complete loss of PS I activity as well as the destabilization of the three peripheral polypeptides, PsaC, PsaD, and PsaE, that are associated with the stromal surface of the PS I complex (5). Complementation studies with the rubA genes of \textit{Synechococcus} sp. PCC 7002 and \textit{Synechocystis} sp. PCC 6803 showed that no unexpected secondary mutations had occurred in the rubA mutant strain. PS II activities in the rubA mutant of \textit{Synechococcus} sp. PCC 7002 were only slightly reduced on a per cell basis relative to the wild-type (76% of wild-type level; Table I). Our observations suggest that RubA is transiently associated with monomeric PS I complexes. Trimerization of PS I complexes requires the PsaL subunit (60), and mutational studies indicate that trimerization is stabilized by the presence of the PsaK subunit. It is likely that trimerization is one of the final steps in PS I biogenesis, since PS I monomers lacking PsaL and PsaK are fully active to perform electron transfer from cytochrome \(c_p\) to flavodoxin (61). Thus, the presence of RubA in the monomer fraction suggests that it might normally exert its action prior to PS I trimerization. In the accompanying paper (58), trimeric PS I complexes from the rubA mutant strain are characterized in detail. These studies establish that the loss of PS I activity results from a specific defect: an inability to assemble the interpeptideyl \([4\text{Fe-4}\text{S}]\) cluster \(F_\text{x}\).

Acknowledgments—We thank Dr. Himadri Pakrasi (Washington University, St. Louis, MO) for providing highly purified samples of plasma and thylakoid membranes from \textit{Synechocystis} sp. PCC 6803, Dr. Wim Vermaas (Arizona State University, Tempe, AZ) for antibodies for the PbcP protein of PS II, and Dr. Donald Kurtz (University of Georgia) for supplying the rubA gene from \textit{C. pasteurianum}.

REFERENCES

1. Golbeck, J. H., and Bryant, D. A. (1991) in \textit{Current Topics in Bioenergetics} (Lee, C. P., ed) Vol. 16, pp. 83–177, Academic Press, Inc., New York. 2. Chitnis, P. R. (2001) \textit{Annu. Rev. Plant Physiol. Plant Mol. Biol.} 52, 593–626. 3. Diner, B. A., and Babcock, G. T. (1990) \textit{In Oxygenic Photosynthesis: The Light Reactions} (Ort, D. R., and Yocum, C. F., eds) pp. 213–247, Kluwer Academic Publishers, Dordrecht, The Netherlands. 4. Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) \textit{Nature} 409, 739–743. 5. Jordan, P., Fromme, P., Witt, H. T., Klukas, O., Saenger, W., and Krauß, N. (2001) \textit{Nature} 411, 809–917. 6. Vassiliev, I. R., Antonkine, M. L., and Golbeck, J. H. (2001) \textit{Biochim. Biophys. Acta} 1507, 139–160. 7. Bartsevich, V. V., and Pakrasi, H. B. (1997) \textit{J. Biol. Chem.} 272, 6382–6387. 8. Boureau, E., Takahashi, Y., Lemieux, D., Turmel, M., and Rochaix, J.-D. (1997) \textit{EMBO J.} 16, 6095–6104. 9. Naver, H., Boudreau, E., and Rochaix, J.-D. (2001) \textit{Plant Cell} 13, 2731–2745. 10. Vermaas, W. F. J. (1993) \textit{Annu. Rev. Plant Physiol. Plant Mol. Biol.} 44, 457–481. 11. Pakrasi, H. B. (1995) \textit{Annu. Rev. Genet.} 29, 755–776. 12. Togasaki R. K., and Surzycki, S. J. (1998) in \textit{The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas} (Rochaix, J.-D.; Golzhedmitz-Clermont, M., and Merchant, S., eds) pp. 13–23, Kluwer Academic Publishers, Dordrecht, The Netherlands. 13. Wasti, J., Duin, E. C., Uznanski, Y., Dierner, W., Link, T., Hoffmann, S., Sticht, H., Dau, H., Lingelbach, K., and Maier, U.-G. (2000) \textit{J. Biol. Chem.} 275, 30058–30063. 14. Shen, G., Antonkine, M. L., Vassiliev, I. R., Golbeck, J. H., and Bryant, D. A. (1998) in \textit{Photosynthesis: Mechanisms and Effects} (Garab, G., ed) Vol. IV, pp. 3147–3150, Kluwer Academic Publishers, Dordrecht, The Netherlands. 15. Stevens, S. E., Jr., and Porter, R. D. (1980) \textit{Proc. Natl. Acad. Sci. U. S. A.} 77, 6052–6056. 16. Shen, G., and Bryant, D. A. (1995) \textit{Photosynth. Res.} 41, 41–53. 17. Buzby, J. S., Porter, R. D., and Stouven, S. E., Jr. (1985) \textit{Science} 230, 805–807. 18. Brosius, J. (1989) \textit{DNA} 8, 759–777. 19. Studier, F. W., Sturewalt, V. L., and Bryant, D. A. (1997) in \textit{Photosynthesis, Biochemistry, and Molecular Biology of Plant Lipids} (Williams, J. P., Khan, M. U., and Lem, N. W., eds) pp. 380–382, Kluwer Academic Publishers, Dordrecht, The Netherlands. 20. Mathieu, I., Meyer, J., and Mouil, J.-M. (1992) \textit{Biochem. J.} 295, 255–262. 21. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorf, J. W. (1990) \textit{Methods Enzymol.} 185, 60–89.

\textsuperscript{4}G. Shen, J. H. Golbeck, and D. A. Bryant, unpublished observations.
