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Expression of a Higher Plant *psbA* Gene in *Synechocystis* 6803 Yields a Functional Hybrid Photosystem II Reaction Center Complex

Peter J. Nixon,[1] Matthias Rögner,[2] and Bruce A. Diner

E. I. du Pont de Nemours and Company, Experimental Station, Central Research and Development Department, P.O. Box 80173, Wilmington, Delaware 19880-0173

The *psbA* gene codes for the D1 polypeptide of the photosystem II reaction center complex and is found in all photosynthetic organisms that carry out oxygenic photosynthesis. Here we describe the construction and characterization of a strain of the cyanobacterium *Synechocystis* sp PCC 6803 in which the three endogenous *psbA* genes are replaced by a single *psbA* gene from the chloroplast genome of the higher plant *Poa annua*. The resulting chimeric strain, KWPAS, grows photoautotrophically with a doubling time of 26 hours compared with 20 hours for wild-type *Synechocystis* 6803. The mutant oxidizes water to oxygen at light-saturated rates comparable with wild type, despite differences in 15% of the primary structure of D1 between these species. RNA gel blot analysis indicates the presence in KWPAS of a *psbA* transcript of approximately 1.25 kilobases, consistent with the chloroplast promoter also acting as a promoter in *Synechocystis*. By using antibodies specific for the carboxyl-terminal extension of the D1 polypeptide of higher plants, we showed that the D1 polypeptide synthesized by KWPAS is post-translationally modified at the carboxyl terminus, probably through processing. A detailed biophysical analysis of the chimeric photosystem II complex indicated that the rates of forward electron transfer are similar to wild type. The rates of charge recombination between the donor and acceptor sides of the reaction center are, however, accelerated by as much as a factor of nine (Q_A^- to S_2) and are the most likely explanation for the lower rate of photoautotrophic growth in the mutant. We conclude that the *psbA* gene from a higher plant can be expressed in cyanobacteria and its product processed and assembled into a functional chimeric photosystem II reaction center.

INTRODUCTION

The photosystem II (PSII) core complex is the membrane protein complex in plants, algae, and cyanobacteria that uses light energy to catalyze the reduction of quinone to quinol and the oxidation of water to oxygen on opposite sides of the thylakoid membrane. The primary light-induced electron transfer reactions of PSII occur within the thylakoid membrane in a heterodimer of two related polypeptides termed D1 (the *psbA* gene product) and D2 (the *psbD* gene product) (Marder et al., 1987; Nanba and Satoh, 1987). The genes encoding these two polypeptides have been characterized from a variety of organisms ranging from prokaryotic cyanobacteria to eukaryotic plants (reviewed in Erickson et al., 1985).

In plants, the *psbA* gene is located in the chloroplast genome as a single uninterrupted copy (e.g., tobacco; Shinozaki et al., 1986), whereas in green algae *psbA* contains introns and may be present as two copies (e.g., *Chlamydomonas reinhardtii*; Erickson et al., 1984). In cyanobacteria, *psbA* is found as a small multicopy gene family of usually three (e.g., *Synechocystis* PCC 6803; Jansson et al., 1987) but sometimes four members (*Anabaena* 7120; Vrba and Curtis, 1989). Despite this variation in gene organization, the D1 polypeptide is relatively highly conserved between species with, for example, an approximately 85% identity between the cyanobacterial and higher plant forms, presumably a reflection of strong functional and structural constraints. The 15% divergence in the primary structure of D1 may reflect a weakening of these constraints but also may be symptomatic of a specific adaptation of the species in performing photosynthesis, such as the interaction of the light-harvesting complexes with PSII or the binding of extrinsic polypeptides necessary for optimal rates of water oxidation. In both of these cases, cyanobacteria differ from green plants and green algae. Instead of the intrinsic light-harvesting chlorophyll *a*/*b*-binding polypeptides of chloroplasts, cyanobacteria use water-soluble extrinsic antennae known as phycobilisomes (reviewed in Glazer, 1989). In addition, cyanobacteria apparently need fewer extrinsic polypeptides for

[1] To whom correspondence should be addressed.

[2] Current address: Westfälische Wilhelms-Universität, Institut für Botanik und Botanischer Garten, Schloßgarten 3, D-4400 Münster, Germany.

the oxidation of water (Stewart et al., 1985). All of these variations may be reflected in sequence differences in the D1 polypeptide.

The most obvious difference in the primary structure of D1 between plants and cyanobacteria is the insertion in cyanobacteria of an extra 7 amino acid residues between residues 347 and 348, close to the carboxyl terminus (for a comparison, see Morden and Golden, 1989). In the higher plant spinach (Takahashi et al., 1988) and the green alga *C. reinhardtii* (B. A. Diner, unpublished results), it is known that D1 undergoes post-translational proteolysis at the carboxyl terminus so that alanine 344 is the terminal amino acid. The reason for such processing is uncertain, and for cyanobacteria there has been until now no evidence in the literature to suggest an equivalent process.

We are interested in studying the structure/function relationships of the D1 polypeptide within PSII through the analysis of specific mutants of the cyanobacterium *Synechocystis* sp PCC 6803, an organism well suited for such experiments (Vermaas, 1988; Williams, 1988). A particular concern was that although the PSII core complex of *Synechocystis* 6803 shows a great similarity in structure and function to the green plant counterpart (Bryant, 1987; Rögner et al., 1990), the differences in sequence of the D1 polypeptide between the species may pose a barrier in fully extrapolating results obtained from the prokaryote to the eukaryotes.

We show that the *psbA* gene from the chloroplast genome of the higher plant *Poa annua*, a festucoid grass, is capable of restoring photosynthetic growth to a *psbA* deletion strain of *Synechocystis* 6803. Our results indicate that the chloroplast gene is transcribed from its own promoter and its product accurately processed and assembled to form a hybrid PSII reaction center. Biophysical analyses of PSII reaction center complexes isolated from this strain indicate that the rates of electron transfer within the hybrid reaction center are similar to those of wild-type centers. These results, therefore, provide compelling evidence in support of the use of *Synechocystis* 6803 as a model system for studying the PSII reaction center. Preliminary aspects of this work were recently presented at the VIIIth International Conference on Photosynthesis in Stockholm (Nixon et al., 1990).

RESULTS

Incorporation of the *psbA* Gene of *P. annua* into the Chromosome of the *psbA* Deletion Strain TD34

The cyanobacterium *Synechocystis* PCC 6803 contains three different copies of the *psbA* gene, termed *psbA1*, *psbA2*, and *psbA3* (Jansson et al., 1987). We have previously described the construction of a deletion strain, TD34, in which the two copies *psbA1* and *psbA2* were replaced by genes conferring resistance to the antibiotics chloramphenicol and kanamycin, respectively, and in which 84% of the coding region of the *psbA3* gene was replaced by a spectinomycin resistance cartridge (Nixon et al., 1990). This strain lacks the D1 polypeptide and so cannot grow photoautotrophically because it cannot synthesize a functional PSII reaction center. TD34 can, however, be grown photoheterotrophically on media supplemented with glucose.

To determine whether the *psbA* gene from the higher plant *P. annua* could restore photosynthetic growth to this strain, we first subcloned a 2.2-kb EcoRI fragment, encompassing the *psbA* gene of *P. annua*, into the integration vector pKW1353 to yield plasmid pKWPAS. Figure 1A shows the insert of plasmid pKWPAS. TD34 was transformed with this construction using standard protocols (Williams, 1988) and transformants were selected for their ability to grow photoautotrophically on BG11 plates. As expected, no PS^+ colonies were obtained when DNA was omitted from the transformation. In contrast, PS^+ colonies were obtained after transformation by pKWPAS at a frequency of about 1×10^{-4} μg^{-1} of DNA per viable cell. This value is typical for transformation of *Synechocystis* 6803 with the vector pKW1353 (Williams, 1988).

A PS^+ transformant, termed KWPAS, was analyzed in greater detail. This strain was capable of growing photosynthetically on chloramphenicol, kanamycin, and spectinomycin, indicating that the three endogenous *psbA* genes were still inactivated. Figure 1B, panel a shows a DNA gel blot hybridization analysis of genomic DNA obtained from TD34 and KWPAS, using an internal fragment of the *psbA* gene from *Amaranthus hybridus* as the probe. Only the *psbA* gene from *P. annua* was detected in KWPAS. The presence of a 1.2-kb BglII/EcoRI-hybridizing fragment (lane 2) demonstrated that the gene was present in an undisturbed form and had not recombined with the small fragment of *psbA3* still left in the genome. The lack of any endogenous *psbA* genes was further confirmed by probing with an internal fragment of the *psbA3* gene at low stringency (result not shown).

A similar blot was probed with the 3.1-kb insert of pKW1353 to check that the *psbA* gene of *P. annua* had indeed been incorporated by way of a double-crossover event (Williams, 1988) into the region of the genome containing this 3.1-kb fragment. The results shown in Figure 1B, panel b confirm that this was so. The probe now hybridizes to a 5.3-kb BamHI fragment from KWPAS, indicating insertion of the 2.2-kb fragment containing the *psbA* gene from *P. annua* into the original 3.1-kb BamHI fragment. In addition, strain KWPAS was sensitive to ampicillin (at 10 μg/mL), further demonstrating that plasmid pKWPAS, which confers resistance to this antibiotic, had not been incorporated into the genome by way of a single crossover event.

These experiments confirmed, therefore, that the photoautotrophic strain KWPAS has incorporated the *psbA*

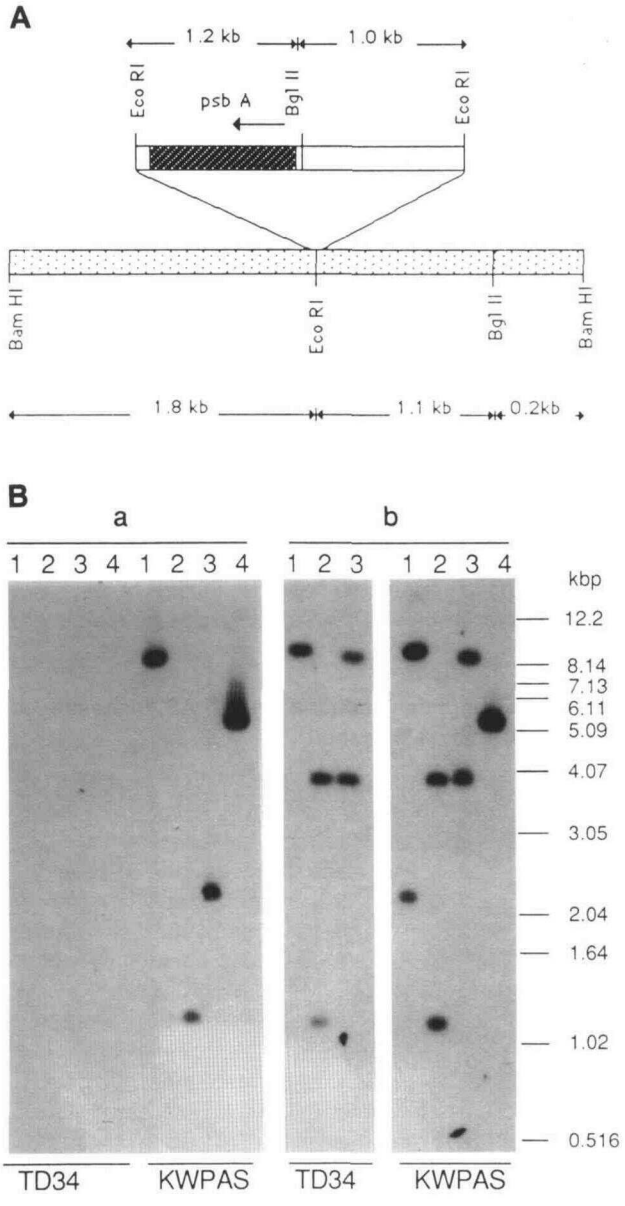

Figure 1. Integration of the *psbA* Gene from *P. annua* into the Chromosome of TD34.

(A) Partial restriction map of the insert from plasmid pKWPAS. The *psbA* gene from *P. annua* is shown in shaded black and DNA from *Synechocystis* 6803 is dotted. The map is not drawn to scale.

(B) DNA gel blot analysis of genomic DNA isolated from TD34 or KWPAS and digested with BglII (lanes 1), BglII/EcoRI (lanes 2), EcoRI (lanes 3), and BamHI (lanes 4). Blot a was probed with *psbA* from *A. hybridus* and blot b with the 3.1-kb BamHI insert of pKW1353. The extents of migration of DNA fragments of known size are shown on the right in kilobase pairs.

gene of *P. annua* into its chromosome and that it is the only copy of *psbA* present.

The *psbA* Gene Product of *P. annua* Is Present in the PSII Core Complex

To show that the *psbA* gene from *P. annua* was indeed expressed in strain KWPAS and its product incorporated into the PSII complex, we used antiserum specific for the D1 polypeptide of higher plants to probe PSII complexes isolated from KWPAS using an HPLC procedure described previously (Rögner et al., 1990). Figure 2 shows immunoblots of the D1 region of a gel, probed with either antiserum specific for the D1 polypeptide of spinach (Vermaas et al., 1988) or antiserum raised to the dodecapeptide ENESANEGYRFG (Diner et al., 1988). This latter antiserum was raised to a portion of the D1 polypeptide that shows a large degree of diversity in primary structure between higher plants and cyanobacteria. For *P. annua*, the equivalent sequence is ENESANEGYKFG (one amino acid change), and in *Synechocystis* 6803 for the *psbA2* and *psbA3* products, it is EVESQNYGYKFG (four amino acid changes).

The difference was such that this antiserum was incapable of recognizing the D1 polypeptide of *Synechocystis* 6803 (Figure 2, left panel). However, it cross-reacted with a diffuse polypeptide in strain KWPAS, which we ascribe to the *psbA* gene product of *P. annua*. Antiserum raised against the D1 polypeptide of spinach recognized D1 in

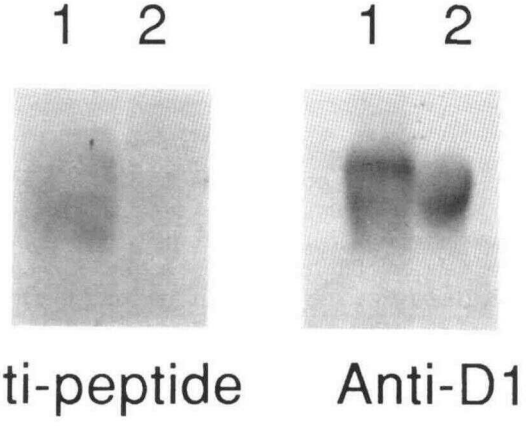

Figure 2. The Product of the *psbA* Gene from *P. annua* Is Present in the PSII Complex of KWPAS.

Immunoblots of PSII core complexes isolated from KWPAS (lanes 1) or the phycocyanin-deficient olive strain (lanes 2) using either an antiserum raised to the peptide ENESANEGYRFG (Anti-peptide) or an antiserum specific for the D1 polypeptide (Anti-D1). Equivalent amounts of chlorophyll were loaded in each track. For clarity, only the 25-kD to 35-kD region of the blot is shown.

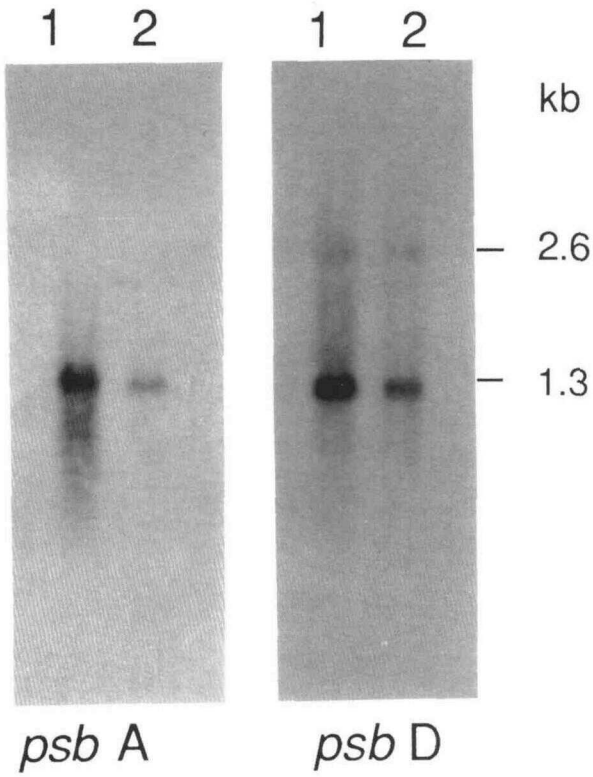

Figure 3. RNA Gel Blot Analysis of *psbA* and *pbsD* Transcripts in KWPAS and WT.

RNA isolated from KWPAS (lanes 1) or WT (lanes 2) was probed with either the *psbA* gene from *A. hybridus* (*psbA*) or the *psbD1* gene from *Synechocystis* 6803 (*psbD*). Approximately 10 μg of RNA was loaded in each track. The approximate sizes of the transcripts as deduced from the migration of RNA molecules of known size are shown in kilobases.

both strains (Figure 2, right panel). However, a greater cross-reaction was obtained against the D1 polypeptide from KWPAS, presumably in part because there is a 99% conservation in primary structure between *P. annua* and spinach and only 85% between spinach and *Synechocystis* 6803. These experiments confirm, therefore, that the *psbA* gene of *P. annua* has been expressed and its product incorporated into the PSII complex.

RNA Gel Blot Analysis of the *psbA* Transcripts in KWPAS

To see how the *psbA* gene of *P. annua* was transcribed in KWPAS, we performed an RNA gel blot hybridization analysis of RNA obtained from either cells of wild type (WT) or KWPAS grown photoautotrophically in BG11 medium. As expected from a previous analysis (Mohamed and Jansson, 1989), Figure 3 shows that the *psbA* genes in WT cells produced in the steady state a message of approximately 1.25 kb (Figure 3, left panel). KWPAS cells also produced a message of similar size but with a greater degree of lower molecular weight smear (Figure 3, left panel, lane 1). No transcript of above 1.3 kb could be detected in this strain, consistent with transcription of the *psbA* gene initiating within the chloroplast-derived fragment. The background smear observed with KWPAS does not appear to be a general problem of RNA degradation because a similar blot probed with an internal fragment of the *psbD1* gene produced a similar hybridization pattern with both WT and KWPAS RNA (Figure 3, right panel). The major mRNA species of about 1.3 kb is probably derived from the *psbD2* gene and the minor transcript of 2.6 kb from the *psbD1/psbC* gene cluster (Mohamed and Jansson, 1989). Therefore, we attribute the more pronounced smear observed with the *psbA* transcripts from KWPAS to either a specific instability of the *psbA* message or a tendency to synthesize prematurely terminated transcripts.

The D1 Polypeptide Produced by KWPAS Possesses a Modified Carboxyl Terminus

In studies on several higher plants (e.g., *Spirodela*; Reisfeld et al., 1982) and green algae (e.g., *Scenedesmus obliquus*; Diner et al., 1988), it has been shown that the D1 polypeptide is synthesized as a precursor polypeptide, which in the cases of *Spirodela* (Marder et al., 1984) and *Scenedesmus* (Diner et al., 1988) is processed within about 2 min of synthesis by the removal of approximately 9 amino acids from the carboxyl terminus. A failure to cleave this precursor in the LF1 mutant of *S. obliquus* has been correlated with an inability to assemble the manganese cluster necessary for water oxidation (Diner et al., 1988; Taylor et al., 1988). Because strain KWPAS synthesizes a D1 polypeptide from a higher plant source and evolves oxygen, this analysis would suggest that processing of D1 also occurs in KWPAS. Alternatively, cyanobacteria such as *Synechocystis* may be different from plants and algae in that processing of D1 is not a prerequisite for water oxidation. Therefore, we investigated whether the carboxyl extension of the D1 polypeptide was still intact in strain KWPAS. To do this, we probed PSII complexes with antibodies specific for a synthetic peptide corresponding to the last 14 amino acids of the *psbA* gene product of spinach, previously shown to be specific for the precursor form of the D1 polypeptide (Diner et al., 1988).

The results in Figure 4 demonstrate that the D1 polypeptide of KWPAS has been modified, probably through processing, so that the antipeptide antibodies fail to cross-react with the *psbA* gene product. To confirm that the

antipeptide antibodies raised against the spinach extension would recognize the precursor form of the D1 polypeptide from *P. annua*, we also probed extracts of *Escherichia coli* that overproduced either β-galactosidase (Figure 4, lanes 4) or a hybrid protein consisting of the complete *psbA* gene product of *P. annua* fused to the carboxyl terminus of β-galactosidase (Figure 4, lanes 3) (Nixon et al., 1987). This fusion resulted in the production of a protein of considerably higher molecular weight than β-galactosidase. The middle panel in Figure 4 shows a control immunoblot using a polyclonal antiserum raised against the D1 polypeptide of spinach. The antiserum recognized the fusion protein, as well as degradation products, and proteins in both the PSII extracts with apparent molecular masses of 30 kD to 34 kD that correspond to D1. The higher molecular mass cross-reaction at approximately 60 kD present with both extracts is an aggregation state of D1 well documented in studies on both plants and cyanobacteria (e.g., Marder et al., 1987; Gounaris et al., 1989). The left panel in Figure 4 shows the results after probing with antibodies specific for the carboxyl-terminal extension. Strong cross-reactions were obtained against the D1 fusion protein and degradation products but none against β-galactosidase (lane 4). This confirmed that these antibodies could also recognize the carboxyl extension of the D1 protein produced by the *P. annua* gene. However, no detectable cross-reaction could be observed against the PSII core complex purified from KWPAS, strongly suggesting that the D1 polypeptide produced in KWPAS had been modified at the carboxyl terminus.

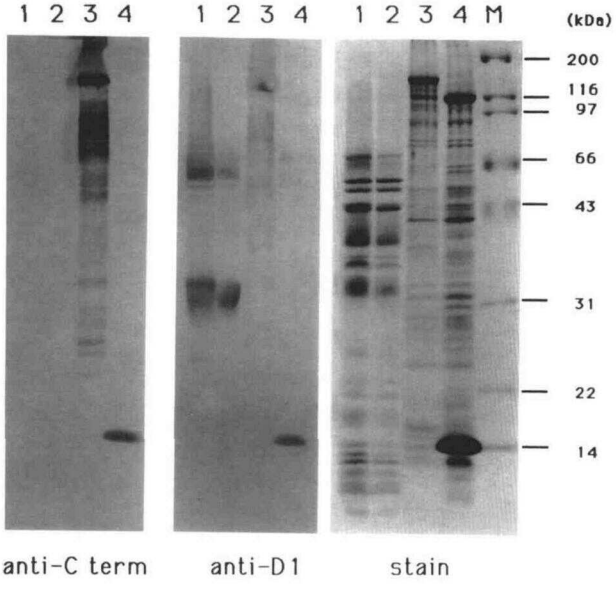

Figure 4. The Carboxyl Terminus of the D1 Polypeptide Is Modified in Strain KWPAS.

An immunoblotting analysis of PSII core complexes from KWPAS (lanes 1) and the phycocyanin-deficient olive strain (lanes 2) together with *E. coli* extracts overproducing either a D1/β-galactosidase fusion protein (lanes 3) or β-galactosidase (lanes 4). One gel was stained with Coomassie Blue (stain) and two others were blotted and immunodecorated with either antibodies specific for the D1 polypeptide of spinach (anti-D1) or specific for a synthetic peptide corresponding to the carboxyl terminus of the D1 polypeptide from spinach (anti-C term). PSII extracts contained approximately 8 μg of chlorophyll. Marker proteins were run in lane M. The band observed at 14 kD in both blots is due to a spurious cross-reaction with the large amount of lysozyme present in lane 4.

Biophysical Characterization of KWPAS

The PSII activity of strain KWPAS was first analyzed by determining the light-saturated rate of oxygen evolution from whole cells grown in the presence of 5 mM glucose. This rate was found to be similar to the WT level of approximately 150 μmol of $O_2 \cdot$mg of chlorophyll$^{-1} \cdot hr^{-1}$. Consistent with this result was the finding that the amount of PSII relative to PSI, determined using the HPLC assay developed by Rögner et al. (1990), was similar in KWPAS to the ratio found in WT cells of 1:10 (on a per-chlorophyll basis). The photoautotrophic growth rate, however, was slightly slower for KWPAS than for WT, with doubling times of approximately 26 hr and 20 hr, respectively (data not shown). Under these growth conditions, KWPAS again showed rates of light-saturated oxygen evolution similar to those of WT and a concentration of PSII reaction center, determined by assaying the amount of the PSII-derived variable fluorescence of chlorophyll from equivalent numbers of cells (data not shown), within 90% of WT levels.

More subtle changes in PSII reaction center activity were determined by examining reactions that involve electron transfer between redox components bound by each of the subunits, D1 and D2. These components include: the primary electron donor, P680, thought to be bound by both D1-histidine 198 and D2-histidine 197 (Michel and Deisenhofer, 1988); the primary and secondary quinone electron acceptors, Q_A and Q_B, respectively, bound by polypeptides D2 and D1, respectively (Diner et al., 1991); the secondary electron donor, Z, corresponding to D1-tyrosine 161 (Debus et al., 1988; Metz et al., 1989); and the oxygen-evolving complex, bound at least in part by polypeptide D1 (Nixon and Diner, 1990; Nixon et al., 1991).

The electron transfer reactions examined were: (1) the quantum yield of photoreduction of Q_A in isolated PSII complexes; (2) the rate of reduction of $P680^+$ by Z and the equilibrium constant governing electron transfer between them; (3) the rates, in whole cells, of electron transfer from Q_A to Q_B, and the equilibrium constant governing the electron transfer between them; and (4) the rate in whole cells of charge recombination between Q_A^- or Q_B^- and the S_2 state of the oxygen-evolving complex, and in PSII core

complexes, the rate of charge recombination between Q_A^- and Z^+. The rates in reaction 4 were then used to estimate the equilibrium constant described for reaction 3. [State S_2 is one of five states of the oxygen-evolving complex (S_0 to S_4), where the subscript refers to the number of stored oxidizing equivalents. States S_2 and S_3 decay in one-electron steps back to S_1, the dark stable state. S_4 decays to S_0, liberating an oxygen molecule (Joliot and Kok, 1975).]

Figure 5 shows the percent photoreduction of Q_A (measured by the absorbance change at 325 nm) as a function of the intensity of laser flashes centered at wavelength 584 nm. No significant differences between core complexes of WT and KWPAS were observed. This experiment indicated that energy transfer from the principal chlorophyll-protein complexes CP47 and CP43 to the reaction center and the primary electron donor P680 is unaltered in the mutant centers. It also showed that reduction of Q_A by $Pheo^-$ (pheophytin *a*), the reduced primary electron acceptor, remains in the mutant very much more rapid than charge recombination between $P680^+$ and $Pheo^-$. Along these same lines, the fluorescence emission spectra of mutant and WT cells, measured at 77 K upon excitation at 580 nm, are similar, indicating equivalent efficiencies of transfer of light energy from the phycobilins to the PSII reaction center (data not shown).

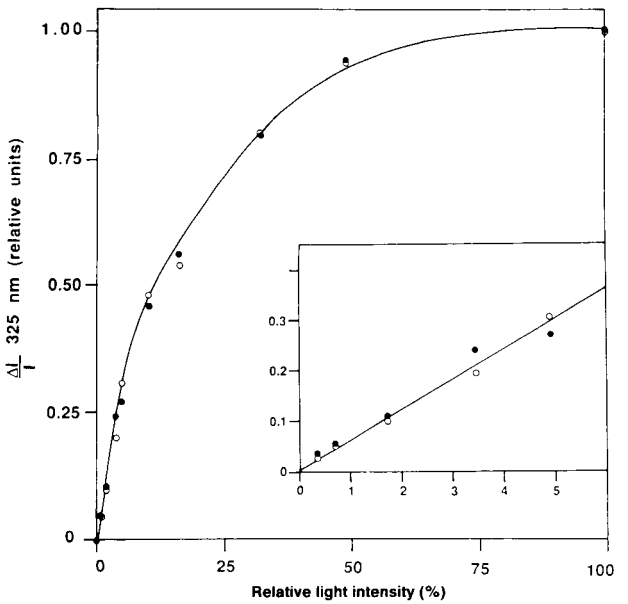

Figure 5. Flash Saturation Curve for the Reduction of Q_A in Mutant and WT Photosystem II Complexes.

PSII core complexes at 10 μM in 20 mM Hepes, pH 7.5, were excited with 600-nsec laser flashes centered at 584 nm (rhodamine 6G in ethanol). The flash-induced absorbance change resulting from Q_A photoreduction was detected at 325 nm, 500 μsec after the actinic flash. The laser flashes were attenuated with neutral density filters calibrated at 584 nm. The mutant and WT curves were normalized to the same amplitude of ΔI/I for the unattenuated actinic flash (100%). ●, WT; ○, KWPAS mutant.

Detection of absorbance changes at 430 nm in isolated core complexes indicated that $P680^+$ from KWPAS is reduced by Z with a $t_{1/2}$ of 10 μsec at pH 5.9 (data not shown), only slightly faster than the 17 μsec observed in WT centers (Metz et al., 1989). Despite this small difference, the ratio of the ΔI/I (430 nm) at 1 μsec, composed solely of $P680^+$, to that at 50 μsec, composed of residual $P680^+$ and Z^+, was equivalent in both centers. Because the relative extinction coefficients of $P680^+$-P680 and Z^+-Z at 430 nm differ by a factor of 15, this observation indicated that the equilibrium $ZP680^+ = Z^+P680$ is similar in mutant and WT centers.

The kinetics of relaxation of the quantum yield of fluorescence after each of two saturating light flashes given to previously dark-adapted cells in state Q_AQ_B are shown in Figure 6A. These measurements are a good indicator of the rate of oxidation of Q_A^- by Q_B, particularly in *Synechocystis* 6803, where there is little indication of energy transfer between PSII centers (Joliot and Joliot, 1964). The kinetics of electron transfer between Q_A and Q_B are similar between the mutant and WT. In the mutant, the reaction $Q_A^-Q_B \rightarrow Q_AQ_B^-$ (after one flash) proceeds with a fast component of $t_{1/2}$ = 125 μsec and in WT with a $t_{1/2}$ of 150 μsec. After the second flash, the kinetics for the reaction $Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$ ($t_{1/2}$, fast component) are 240 μsec in the mutant and 490 μsec in WT. The difference in rates between the first and second flashes is consistent with an earlier observation by Robinson and Crofts (1983) that the formation of the Q_B quinol is slower than the formation of the Q_B semiquinone. The quinol Q_BH_2 dissociates from the center and is replaced by quinone, Q_B, regenerating the initial state. On every other flash, therefore, the system finds itself in the same state. Consequently, the rate of oxidation of Q_A^- should oscillate with a period of two flashes, as is borne out by the measurement shown in Figure 6B of the quantum yield of fluorescence detected 750 μsec after each of a series of five actinic flashes (1.67 Hz).

Q_B^- can be generated by giving a single preflash to dark-adapted cells (in the fully oxidized state Q_AQ_B). By waiting a variable time before starting the flash series, it is possible, by analyzing the dephasing of the oscillations of period two (shown in Figure 6B) as a function of time, to determine the rate of recombination between donor state S_2 and Q_B^- (Robinson and Crofts, 1983). This type of analysis gives a single exponential decay for Q_B^- oxidation with a $t_{1/2}$ of 15 ± 6 sec in the mutant cells and a $t_{1/2}$ of 47 ± 7 sec for WT cells (data not shown). The ratio of this rate to the rate of recombination between Q_A^- and S_2, measured in the presence of the inhibitor DCMU, gives the equilibrium constant $K_{AB(1)}$, plus 1, where *K* describes the equilibrium $Q_A^-Q_B = Q_AQ_B^-$. Figure 7 shows the kinetics of charge recombination between Q_A^- and state S_2 in whole cells in the pres-

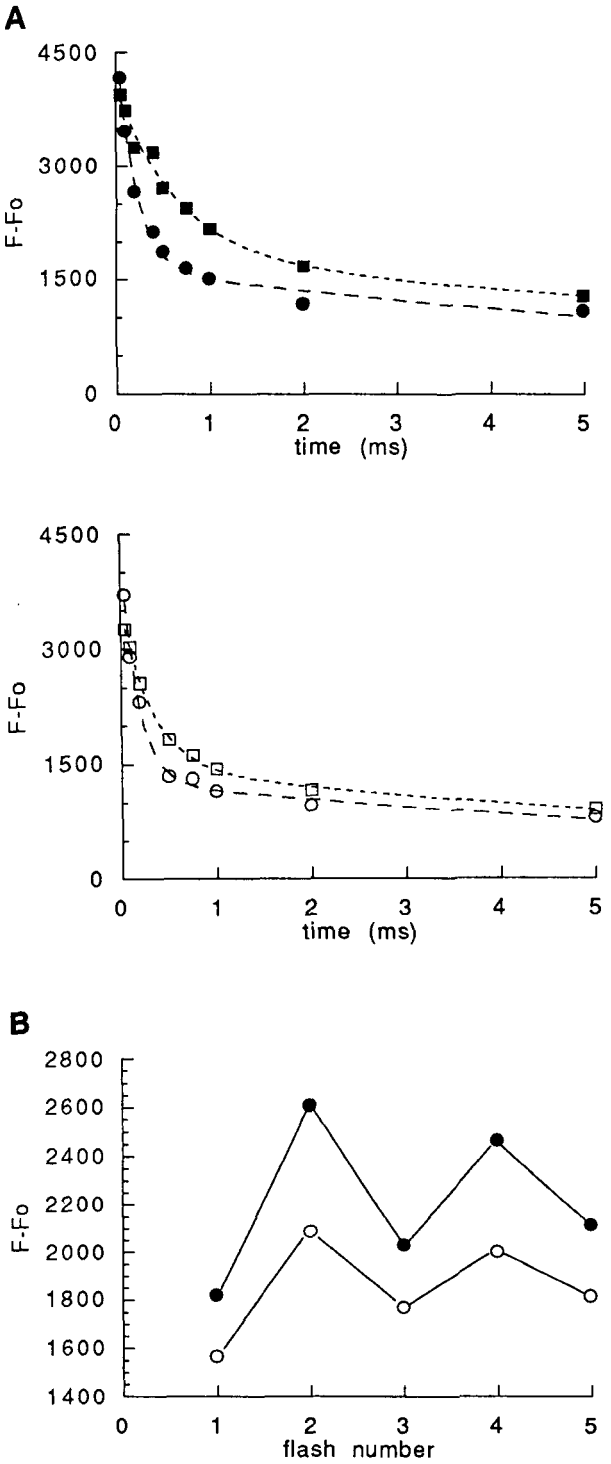

Figure 6. Measurement of the Rates of Relaxation of the Quantum Yield of Chlorophyll Fluorescence after Saturating Flash Excitation.

(A) Mutant and WT cells were pretreated with *p*-benzoquinone and ferricyanide in the dark in BG-11 medium plus 50 mM Hepes, pH 7.5, as described in Methods, to oxidize the primary and secondary quinone electron acceptors to the Q_AQ_B state. Two saturating microsecond flashes, given 600 msec apart, were followed by a series of probe flashes at the indicated times and at 422 nm to monitor the variation in the quantum yield of chlorophyll fluorescence, detected >660 nm. The fluorescence yield preceding the actinic flash (Fo) is subtracted from that following the flash (F) and plotted accordingly as F-Fo. F-Fo is proportional to the concentration of reduced Q_A and is maximal at the earliest time point shown, 50 μsec after the actinic flash. Top, WT; ●, first flash; ■, second flash. Bottom, KWPAS mutant; ○, first flash; □, second flash.

(B) F-Fo was detected at 750 μsec after each of a series (1.67 Hz) of saturating actinic flashes in mutant and WT cells. The conditions of the experiment are the same as in **(A)**. ●, WT; ○, KWPAS mutant.

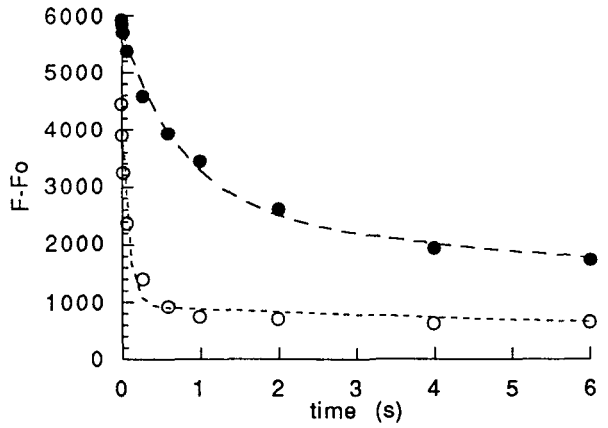

Figure 7. Relaxation of the Quantum Yield of Fluorescence in the Presence of DCMU after a Single Saturating Actinic Flash.

Except for the presence of 40 μM DCMU, the conditions for this experiment are the same as in Figure 6. ●, WT; ○, KWPAS mutant. (Fmax – Fo)/Fo were 0.54 and 0.37 for WT and KWPAS, respectively, in the experiments of Figures 6 and 7.

ence of DCMU, measured using the same fluorescence method as above. S_2/Q_A^- charge recombination to give the state S_1/Q_A was found to occur with a $t_{1/2}$ (for the fast component) of 70 msec and 600 msec in the mutant and WT, respectively (Figure 7). Together, these measurements imply a value of $K_{AB(1)}$ some threefold higher in the mutant than in WT.

An examination of charge recombination between Q_A^- and Z^+ in core complexes at pH 5.9 indicated a $t_{1/2}$ of about 20 msec for the mutant and 70 msec for WT (data not shown). Both this measurement and that described in the previous paragraph can be explained by a small negative shift in the mutant compared with the wild type of 20 mV to 30 mV in the midpoint potential of Q_A/Q_A^- and a small positive shift of 20 mV to 30 mV for S_2/S_1 of the oxygen-evolving complex. These shifts would cause an accelera-

tion of the rates of charge recombination by way of a reverse of forward electron flow through pheophytin.

DISCUSSION

In this paper we have shown that the *psbA* gene from the chloroplast genome of the monocot *P. annua* can substitute for the *psbA* gene family of the cyanobacterium *Synechocystis* PCC 6803 to produce a strain that synthesizes a hybrid PSII core complex. The biophysical properties of this complex are similar to those of the wild type. No attempt was made to use a cyanobacterial promoter to drive transcription of the chloroplast gene; instead, we relied on the chloroplast promoter to function in *Synechocystis*, a reasonable assumption because an earlier study had already shown that the *psbA* promoter from *A. hybridus* could function in the cyanobacterium *Synechococcus* PCC 7942 (Dzelzkalns et al., 1984). The RNA gel blot experiment shown in Figure 3 argues that transcription does indeed initiate from the chloroplast *psbA* promoter. This observation, therefore, supports the idea that promoters that function in chloroplasts may also be active in *Synechocystis* 6803. Recent support for this proposal has also come from the observation by Los et al. (1990) that DNA fragments from the chloroplast genome of the alga *Dunaliella salina* are transcriptionally active in *Synechocystis* 6803.

Processing of the D1 Polypeptide

An important consequence of this work is the finding that the carboxyl terminus of the D1 polypeptide found in the PSII complex of KWPAS is modified so that antibodies specific for the last 14 amino acids of the *psbA* gene product no longer recognize the mature polypeptide. This observation would indicate that D1 has undergone proteolysis at the carboxyl terminus in a manner analogous to that found in green plants and that, with the possible exception of the alga *Euglena gracilis* (Karabin et al., 1984; Keller and Stutz, 1984), post-translational processing of the D1 polypeptide is ubiquitous among photosynthetic organisms. Indeed, we have recently sequenced tryptic peptides derived from WT PSII core complexes and found that the D1 polypeptide of *Synechocystis* 6803 is also cleaved after alanine 344 (P.J. Nixon and B.A. Diner, unpublished results).

Little is known about the substrate specificity of the protease responsible for processing D1. The results presented here would suggest that the protease present in *Synechocystis* 6803 can recognize and cleave a higher plant D1 precursor molecule, even though there is a high degree of divergence between cyanobacteria and green plants in the primary structure of the carboxyl-terminal region of D1. In support of this idea of broad substrate specificity is the recent work demonstrating that the purified processing enzyme from both spinach (Inagaki et al., 1990) and pea (Packer et al., 1990) can process the D1 polypeptide found in the LF1 mutant of the green alga *S. obliquus*, despite a conservation of only 5 out of 9 residues (Todd et al., 1990) after the proposed cleavage site on the carboxyl side of alanine 344. Presumably, sequences within the mature portion of the D1 polypeptide are more important for recognition by the protease.

Biophysical Analyses—Similarities

Measurements of photoreduction of Q_A as a function of flash intensity on purified WT and chimeric PSII complexes indicate equivalent quantum yields of charge separation. This means that energy transfer between CP47 and CP43 and the reaction center is unaffected by exchange of *Synechocystis* D1 with that of *P. annua*. It also means that electron transfer from $Pheo^-$ to Q_A remains much more rapid than recombination between $Pheo^-$ and $P680^+$. In addition, fluorescence emission spectra of mutant and WT cells, measured at 77 K upon excitation at 580 nm, are similar, indicating equivalent efficiencies of transfer of light energy from the phycobilins to the PSII reaction center (data not shown). These observations support the idea that the sequence differences in D1 between *Synechocystis* 6803 and *P. annua* have little effect on the harvesting of light energy by the phycobilisomes and transfer of that light energy to the PSII reaction center.

Reduction of $P680^+$ by Z in the mutant is only slightly accelerated ($t_{½}$ = 10 μsec) relative to WT ($t_{½}$ = 17 μsec) at pH 5.9, and the equilibrium on the donor side, $ZP680^+ = Z^+P680$, is unaffected. These observations are not surprising because the amino acid residues in D1 and D2 that are thought to be close to these redox-active species are highly conserved between species (Michel and Deisenhofer, 1988).

The rates of electron transfer between the D1 and D2 subunits of the reaction center are an indication of how well these fit together after substitution of D1. Electron transfer between the quinones is such an indicator because Q_A is bound by polypeptide D2 and Q_B by D1. Despite the small apparent differences in the energetics of acceptor side electron transfer in the KWPAS centers, the kinetics of forward electron transfer between the quinones are practically unaltered (Figure 6A). The two-electron gate that governs the reduction of quinone, Q_B, to the quinol in two successive electron transfer steps is also functioning normally, as evidenced by the oscillation of period two (Figure 6B) in the rates of electron transfer for $Q_A^-Q_B \rightarrow Q_AQ_B^-$ (fast) and $Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$ (slow) (Robinson and Crofts, 1983). This similarity would imply that protonation steps coupled to electron transfer are not altered in the mutant. The ability of D1 to bind Q_B also appears unaltered.

Biophysical Analyses—Differences

The more significant but still minor differences between KWPAS and WT that we have detected concern principally

the rates of charge recombination between Q_A^- and Q_B^- and the S_2 state of the oxygen-evolving complex in whole cells and between Q_A^- and Z^+ in core complexes. We find that the equilibrium constant for $Q_A^-Q_B = Q_AQ_B^-$ is approximately threefold higher in mutant cells, corresponding to a negative shift of the midpoint potential of Q_A/Q_A^- of 20 mV to 30 mV relative to Q_B/Q_B^-. At the same time, the more rapid recombination between Q_A^- and Q_B^- and the donor side implies a lowering of the activation energy for charge recombination, which probably proceeds at room temperature by way of the reformation of $P680^+$-$Pheo^-$, an intermediate in forward electron transfer. Such a decrease in the activation energy would likely arise from a decrease in the free energy difference between redox couples Pheo/$Pheo^-$ and Q_A/Q_A^-, with the difference between Pheo/$Pheo^-$ and Q_B/Q_B^- remaining constant. On a fixed redox scale, either redox couple Q_A/Q_A^- has shifted negative or both Pheo/$Pheo^-$ and Q_B/Q_B^- have shifted positive to an equal extent with respect to Q_A/Q_A^-. It is likely that these shifts in the redox couples of the acceptor side arise from the formation of a hybrid center rather than from any intrinsic differences of *P. annua* D1. The equilibria and Q_A^--S_2 recombination rates are similar in *Synechocystis* cells and in spinach (Diner, 1977; Robinson and Crofts, 1984), with the primary structure of D1 in the latter almost completely conserved with respect to *P. annua*. In *P. annua*, glutamate 11 and arginine 238 of spinach are replaced by threonine and lysine, respectively (Barros and Dyer, 1988).

The rates and equilibria observed in the chimeric mutant, while not as extreme, are reminiscent of differences observed between the reaction centers of the purple nonsulfur photosynthetic bacteria *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides*. *Rps. viridis* reaction centers have a 10-fold to 50-fold higher equilibrium constant for the reaction $Q_A^-Q_B = Q_AQ_B^-$ and a 100-fold more rapid charge recombination between Q_A^- and oxidized primary donor P^+ (Shopes and Wraight, 1985, 1987). These differences arise in large part from a 75-mV to 100-mV negative shift in the midpoint potential (E_{m7}) of Q_A/Q_A^- in chromatophores of *Rps. viridis* as compared with *Rb. sphaeroides* as measured by redox titration (Cogdell and Crofts, 1972; Prince and Dutton, 1978; Rutherford et al., 1979). From the equilibrium constants, the E_m of Q_B/Q_B^- would be expected to be similar for the two. The lower E_m for Q_A/Q_A^- arises in part from the presence of the lower potential menaquinone in the Q_A binding site in *Rps. viridis* compared with ubiquinone in the corresponding site in *Rb. sphaeroides*, but mostly from an intrinsic difference in the protein portion of the reaction centers that lowers further the midpoint potential of Q_A/Q_A^- in *Rps. viridis* (Shopes and Wraight, 1985).

Overall, the differences we observed in the energetics of the primary and secondary electron transfer reactions in the hybrid reaction center are of minor consequence for the photosynthetic viability of the mutant, just as for *Rps. viridis* and *Rb. sphaeroides*, both of which are found in nature. There is no detectable difference in the quantum yield of charge separation, and the mutant and WT cells remain similar in their rates of light-saturated oxygen evolution. These observations are consistent with the work of Gunner and Dutton (1989), who replaced ubiquinone in the Q_A site by a large number of quinones in *Rb. sphaeroides* reaction centers. They showed that such replacement had little impact on the quantum efficiency of primary charge separation until the free energy difference ($-\Delta G°$) between redox couples Pheo/$Pheo^-$ and Q_A/Q_A^- dropped to close to 400 meV from 650 meV with ubiquinone in the Q_A site.

There are, however, differences in the growth rates of the KWPAS mutant and WT measured under photoautotrophic conditions: doubling times of 26 hr and 20 hr, respectively. This difference likely arises from the threefold more rapid rate of charge recombination between Q_B^- and the S_2 state, which would compete with light-driven charge separation in the reaction center at moderate light intensities.

Other Attempts at Gene Substitution

The *psbA* gene from *P. annua* is not the only higher plant *psbA* gene capable of restoring photosynthetic growth to a strain of *Synechocystis* 6803 that lacks functional *psbA* genes. Reiss et al. (1990) have reported recently the incorporation of the *psbA* gene from *A. hybridus* into the chromosome of a strain of *Synechocystis* 6803 in which the *psbA* gene family had been inactivated by insertional mutagenesis. The resulting strain grew photoautotrophically. However, care must be taken in experiments that use host strains containing interrupted endogenous genes because the active recombination pathways found in *Synechocystis* 6803 may generate a hybrid *psbA* gene consisting of portions of both the higher plant and cyanobacterial genes.

Attempts have also been made to replace the *psbB* and *psbC* genes of *Synechocystis* 6803 with their higher plant homologs. In all cases, the mutants are nonphotosynthetic (Vermaas et al., 1989; Carpenter et al., 1990). In the case of the *psbC* gene, which encodes the apopolypeptide of the chlorophyll/protein complex CP43, it appears that cyanobacterial portions of the *psbC* gene are required for accumulation of CP43 in the membrane (Carpenter et al., 1990). Therefore, higher plant genes cannot be assumed to be indiscriminately interchangeable with their cyanobacterial counterparts.

Cyanobacteria such as *Synechocystis* 6803 and higher plants such as *P. annua* are thought to have diverged about 800 to 2000 million years ago (Wilson et al., 1987). The ability of the *psbA* gene from *P. annua* to replace the equivalent cyanobacterial genes highlights the strong structural and functional constraints placed on the D1 polypeptide, consistent with its key role in transmembrane charge separation from donor Z to the secondary quinone Q_B, as well as possibly binding part or all of the manganese cluster (Nixon and Diner, 1990; Nixon et al., 1991).

METHODS

Bacterial Strains and Growth Conditions

Synechocystis *PCC 6803*

The glucose-tolerant strain, referred to here as the wild type, (Williams, 1988); the phycocyanin-deficient olive strain (Rögner et al., 1990), and the deletion strain TD34, which lacks all three copies of the *psbA* gene (Nixon et al., 1990), have been described previously. All strains were grown as described by Williams (1988) in either liquid medium or on agar plates under constant illumination (about 3 W/m^2 using cool-white fluorescent lamps) at 30°C. Glucose at 5 mM was included in the media when photoheterotrophic growth was required. The antibiotics chloramphenicol (5 μg/mL), kanamycin (50 μg/mL), and spectinomycin (50 μg/mL) were used at the levels indicated. Growth of cyanobacterial cultures was estimated by measuring the scattering of light at 730 nm using a Hewlett Packard diode array spectrophotometer.

Escherichia coli

Strain XL1-blue [*endA1*, *hsdR17*, (r_k^-, m_k^+), *supE44*, *thi*-1, λ^-, *recA1*, *gyrA96*, *relA1*, (*lac*$^-$), (F′, *proAB*, *lacIqZ*delM15, Tn10 [tetR])], supplied by Stratagene was used as the host for all cloning experiments. *E. coli* was grown in LB medium as described (Maniatis et al., 1982) and in the presence of ampicillin at 100 μg/mL unless stated otherwise.

Plasmids

Plasmid pPAS 3-91, which contains a 2.2-kb EcoRI fragment of chloroplast DNA from *Poa annua* cloned into pUC9, was used as a convenient source of the higher plant *psbA* gene (Barros and Dyer, 1988). The integration vector pKW1353 is a derivative of pKW1188 (Williams, 1988) and consists of a 3.1-kb BamHI fragment of *Synechocystis* 6803 genomic DNA cloned into the BamHI site of a pUC9 derivative (Williams, 1988). Plasmid pKWPAS, used to transfer the *psbA* gene of *P. annua* into the chromosome of *Synechocystis* 6803, was constructed by subcloning the 2.2-kb EcoRI fragment of pPAS 3-91 into the unique EcoRI site of pKW1353. The construction of plasmid pPND1, used to express the *psbA* gene product of *P. annua* in *E. coli* as a β-galactosidase fusion protein, has been described (Nixon et al., 1987). Plasmid pUR292, which directs the overproduction of β-galactosidase in *E. coli* and from which pPND1 is derived, is one of a set of expression vectors (Rüther and Müller-Hill, 1983).

Molecular Cloning

All cloning procedures used were essentially those described by Maniatis et al. (1982). Enzymes used to modify DNA were purchased from Promega Biotec and used according to their instructions.

Preparation and Analysis of RNA

RNA was extracted from cells grown photoautotrophically to late log phase using the hot phenol method described by Mohamed and Jansson (1989). RNA was separated on 37% (v/v) formaldehyde gels and transferred to GeneScreen-*Plus* nylon membranes according to the manufacturer's instructions (New England Nuclear Research Products). RNA size markers (9.49 kb, 7.46 kb, 4.40 kb, 2.37 kb, 1.35 kb, and 0.24 kb) were purchased from Bethesda Research Laboratories. The filters were prehybridized at 42°C for 20 hr, and then hybridized with the radiolabeled probe at 42°C for 24 hr in 50% (v/v) formamide, 5 × SSC (where 20 × SSC is 0.3 M sodium citrate, pH 7.5, 3 M NaCl), 50 mM NaPi, pH 6.5, 250 μg/mL denatured salmon sperm DNA, 0.02% (w/v) BSA, 0.02% (w/v) Ficoll, and 0.02% (w/v) polyvinylpyrolidone. Filters were washed at room temperature four times with 2 × SSC and 1% SDS, then twice with 0.1 × SSC.

Preparation and Analysis of DNA

Genomic DNA was obtained from 50-mL cultures of *Synechocystis* 6803 using the procedure outlined by Williams (1988). DNA probes were radiolabeled using the random-primer labeling method of Feinberg and Vogelstein (1983) and consisted of a 1.0-kb BamHI/XbaI fragment from plasmid pKW1213 that contained an internal portion of the *psbA* gene of *Amaranthus hybridus*; a 432-bp KpnI fragment from *Synechocystis* 6803 containing part of the *psbA3* gene (Metz et al., 1989); and a 0.8-kb SauI/XbaI fragment containing part of the *psbD1* gene from *Synechocystis* 6803 (Williams and Chisholm, 1987).

Polyacrylamide Gel Electrophoresis and Immunoblotting

Proteins were separated under denaturing conditions on 12% (w/v) polyacrylamide gels (in the absence of urea) using the procedure described by Rögner et al. (1990) and were visualized by staining with Coomassie Brilliant Blue R-250. Molecular weight markers were purchased from Bio-Rad and consisted of myosin (200 kD), β-galactosidase (116 kD), phosphorylase *b* (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

Immunoblotting was performed as described previously (Nixon et al., 1989).

Antibodies

Antiserum raised against the D1 polypeptide of spinach was kindly provided by Wim Vermaas (Vermaas et al., 1988). The production of antiserum to the tetradecapeptide PLDLAAIEAPSTNG, corresponding to the unprocessed carboxyl terminus of the D1 polypeptide from spinach, and the dodecapeptide ENESANEGYRFG, corresponding to amino acids 229 through 240 of the same polypeptide, has already been reported (Diner et al., 1988). Antibodies specific for *E. coli* proteins were removed by treatment of the crude antiserum with protein lysates of *E. coli* (XL1-blue) that had been coupled to an Affi-Gel support according to the manufacturer's instructions (Bio-Rad). Antibodies specific for the D1/β-galactosidase fusion protein were purified using a variation of the chromatography procedure described by Nivison and Hanson (1987). Briefly, an affinity matrix was constructed by coupling approximately 1 mg of gel-purified fusion protein to approximately 4 mL of Affi-Gel (a 1:1 combination of Affi-Gel 10 and Affi-Gel 15, Bio-Rad) in coupling buffer (0.1 M Mops, 1% SDS, pH 8.0), according to the manufacturer's instructions. Two hundred milliliters of crude antiserum was diluted by the addition of an equal volume of 2× starting buffer [0.2 M KPi, 1.0 M NaCl, 1% (v/v) Triton X-100, pH 7.0] and incubated with 400 μL of the affinity matrix at 4°C for 20 hr in a 1.5 mL microcentrifuge tube. The gel

was washed with 1× starting buffer (6 × 1 mL) and then with 0.5 M NaCl (3 × 1 mL). Affinity-bound antibodies were released by 0.1 M glycine, pH 2.45 (3 × 200 μL), and quickly neutralized in 105 μL of 1 M Tris, pH 7.5.

Preparation of *E. coli* Extracts

E. coli (XL1-blue) containing either pPND1 or pUR292 were grown in LB medium containing ampicillin (50 μg/mL). Isopropyl-thio-β-D-galactopyranoside (1 mM) was added to induce synthesis of the D1/β-galactosidase fusion protein or β-galactosidase. Cells were harvested and solubilized in 3 × solubilizing buffer [187 mM Tris HCl, pH 6.8, 9% (w/v) SDS, 15% (v/v) β-mercaptoethanol, 30% (v/v) glycerol]. Occasionally, as in the case of those carrying plasmid pUR292, cells were treated with an excess of lysozyme before the solubilizing buffer to aid solubilization.

Preparation of PSII Core Complexes and Preoxidized Cells from *Synechocystis* 6803

PSII nonoxygen-evolving core complexes were obtained by anion-exchange chromatography of detergent-solubilized thylakoid membranes using HPLC (Rögner et al., 1990). Complexes isolated from the olive strain are identical to those isolated from WT. The samples analyzed were obtained after only one Mono Q chromotography step, rather than the usual two, and therefore contain several impurities, notably the ATP-synthetase complex.

Before fluorescence kinetic measurements, mutant and WT cells (see below) were treated at a concentration of 0.9 OD at 730 nm for 15 min with 0.4 mM *p*-benzoquinone and 0.4 mM $K_3Fe(CN)_6$ in BG-11 medium containing 50 mM Hepes, pH 7.5. The cells were pelleted for 5 min at 3000*g* and resuspended in BG-11 medium plus 50 mM Hepes, pH 7.5, at the same cell concentration in the absence of benzoquinone and ferricyanide. All manipulations were performed in the dark at 25°C.

Functional Measurements

The rates of charge recombination between Q_A^- and Z^+ and of reduction of $P680^+$ by Z were measured in PSII core complexes by monitoring absorbance changes at 325 nm and 432 nm, respectively, in a flash detection spectrophotometer (Metz et al., 1989) similar to an instrument described by Joliot et al. (1980). Flash saturation curves for photoreduction of Q_A were also performed on PSII core complexes at 325 nm. Actinic flashes (600 nsec duration) were provided by a pulsed dye laser (SLL-250, Candela, Wayland, MA) containing rhodamine 6G (λ_{max} = 584 nm) and attenuated using neutral density filters.

This same instrument was used for measurements of the rates of oxidation of Q_A^- in whole cells by monitoring the variation in quantum yield of chlorophyll fluorescence after an actinic flash. Q_A^- is oxidized by electron transfer to Q_B, the secondary quinone acceptor, and by charge recombination with the donor side in the absence and presence of DCMU. The actinic flash was filtered through an infrared reflecting filter (MTO Athervex TA2, Massy, France), a Corning 4-96 filter, and a Kodak Wratten 34 filter. The fluorescence yield was measured with probe flashes at 422 nm. The sample photodiode was protected by a set of blocking filters—an infrared reflecting filter (MTO Athervex TA2), Schott low fluorescence filters KV470 and KV550, and a Corning 2-64 filter, together transmitting >660 nm.

Rates of light-saturated oxygen evolution at 25°C were measured using a Clark-type oxygen electrode (model DW1, Hansatech, King's Lynn, Norfolk, England). Whole cells were assayed in liquid BG11 medium plus 5 mM glucose to which were added 1 mM potassium ferricyanide and 0.3 mM 2,6-dichloro-*p*-benzoquinone.

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