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Targeted genetic inactivation of the photosystem I reaction center in the cyanobacterium *Synechocystis* sp. PCC 6803

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We describe the first complete segregation of a targeted inactivation of *psaA* encoding one of the P_{700}-chlorophyll *a* apoproteins of photosystem (PS) I. A kanamycin resistance gene was used to interrupt the *psaA* gene in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Selection of a fully segregated mutant, ADK9, was performed under light-activated heterotrophic growth (LAHG) conditions; complete darkness except for 5 min of light every 24 h and 5 mM glucose. Under these conditions, wild-type cells showed a 4-fold decrease in chlorophyll (chl) per cell, primarily due to a decrease of PS I reaction centers. Evidence for the absence of PS I in ADK9 includes: the lack of EPR (electron paramagnetic resonance) signal I, from P_{700}^+; undetectable P_{700}-apoprotein; greatly reduced whole-chain photosynthesis rates; and greatly reduced chl per cell, resulting in a turquoise blue phenotype. The PS I peripheral proteins PSA-C and PSA-D were not detected in this mutant. ADK9 does assemble near wild-type levels of functional PS II per cell, evidenced by: EPR signal II from Y_D^+; high rates of oxygen evolution with 2,6-dichloro-*p*-benzoquinone (DCBQ), an electron acceptor from PS II; and accumulation of D1, a PS II core polypeptide. The success of this transformation indicates that this cyanobacterium may be utilized for site-directed mutagenesis of the PS I core.

Key words: EPR spectroscopy/heterotrophic growth/photosystem I/*psaA*/targeted mutagenesis

Introduction

Photosystem (PS) I is a multi-subunit, membrane-bound protein complex that functions as a light-driven, plastocyanin: ferredoxin oxidoreductase. PS I operates in both cyclic electron flow, driving ATP synthesis, and in linear electron flow, which drives ATP synthesis and the generation of reducing power as NADPH. Light energy is captured by the PS I antenna and is funneled to a chlorophyll (chl) *a* special-pair, P_{700}, which passes an energized electron to A_0 (a specialized chl *a*). P_{700}^+ is then reducd by plastocyanin, which may be replaced by cytochrome c_{553} in cyanobacteria (Sandmann and Böger, 1980). Electron transfer proceeds from A_0 to A_1 (most likely a phylloquinone), and then to F_x (a [4Fe-4S] center), which donates electrons to F_A and/or F_B (both [4Fe-4S] centers), the terminal acceptors of PS I. F_A and/or F_B reduce the soluble electron carrier ferredoxin, which reduces $NADP^+$ to NADPH, catalyzed by the ferredoxin:$NADP^+$ oxidoreductase (for a recent review of PS I, see Golbeck and Bryant, 1991). The electron transfer components P_{700}, A_0, A_1 and F_x are bound to a heterodimer of homologous 82-83 kDa polypeptides, PSA-A and PSA-B (Golbeck *et al.*, 1988; Golbeck and Bryant, 1991), encoded by the *psaA* and *psaB* genes, which have been mapped to the plastome of plants and algae (Westhoff *et al.*, 1983; Girard-Bascou *et al.*, 1987). Centers F_A and F_B are bound to a 9 kDa polypeptide, PSA-C, also encoded in the plastome by *psaC* (Høj *et al.*, 1987). Functions have been proposed for two of the eight additional known PS I proteins: PSA-D is thought to be involved in ferredoxin binding (Zilber and Malkin, 1988), while PSA-F may be involved in plastocyanin binding (Wynn and Malkin, 1988).

Mutants with aberrant PS I have been isolated in *Chlamydomonas reinhardtii* (Chua *et al.*, 1975; Girard *et al.*, 1980; Girard-Bascou, 1987), maize (Miles *et al.*, 1979; Cook and Miles, 1990), barley (Møller *et al.*, 1980), *Antirrhinum majus* (Herrmann, 1971), *Scenedesmus* (Gregory *et al.*, 1971) and *Oenothera* (Fork and Heber, 1968). Two nuclear mutants of *C.reinhardtii* described by Chua *et al.* (1975) lack P_{700} activity and the P_{700}-apoproteins, yet still maintain wild-type levels of PS II activity and PS II core proteins. Further analysis of those mutants and 23 other nuclear mutants of *C.reinhardtii* deficient in the PS I reaction center proteins revealed the loss of a group of small polypeptides that are enriched in PS I preparations from wild-type cells (Girard *et al.*, 1980). Some of those nuclear mutations have been shown to block the *trans*- splicing of the *psaA* message in the chloroplast, thus abolishing PS I assembly (Choquet *et al.*, 1988; Goldschmidt-Clermont *et al.*, 1990). In addition, mutations mapped to the *psaB* gene in the plastome of *C.reinhardtii* result in the loss of the PS I reaction center, as well as the same group of small polypeptides deficient in the nuclear mutants (Girard-Bascou *et al.*, 1987). A nuclear mutant of barley, deficient in the PS I reaction center, also lacks at least two small proteins that are enriched in wild-type PS I particles, but maintains wild-type levels of PS II activity (Møller *et al.*, 1980). Plastome mutants of *Oenothera* (Fork and Heber, 1968) and *Antirrhinum* (Hermann, 1971) and nuclear mutants of maize (Miles *et al.*, 1979) that are deficient in the PS I reaction center do have PS II activity.

Targeted mutagenesis of PS I genes has not been accomplished in plants or algae; however, inactivation of genes for two peripheral PS I proteins has been achieved in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Chitnis *et al.*, 1989a,b). Inactivation of the *psaD* gene resulted in reduced growth rate under photoautotrophic conditions, but wild-type growth rate under photoheterotrophic conditions {with DCMU [N_1-(dichlorophenyl)-N_3-dimethylurea] and glucose in the light} (Chitnis *et al.*, 1989a). When the *psaE* gene, encoding an 8 kDa protein

with unknown function, was inactivated, only minor changes in PS I activity and photoautotrophic growth were observed (Chitnis *et al.*, 1989b). Similar results were obtained when *psaE* was inactivated in the cyanobacterium *Synechococcus* sp. PCC 7002 (Bryant *et al.*, 1990).

Attempts at targeted mutagenesis of the *psaA-psaB* genes in *Synechococcus* sp. PCC 7002 have resulted in only partial segregation of the mutation (Zhao *et al.*, 1990), perhaps because selection was performed in the light, which gave a selective advantage to cells maintaining wild-type PS I. This problem is avoided by performing selection and segregation under heterotrophic growth conditions, which have recently been defined for *Synechocystis* 6803 (Anderson and McIntosh, 1991). Termed light-activated heterotrophic growth (LAHG), cells are grown with 5 mM glucose in complete darkness except for 5 min of light (40 μmol/m^2/s) every 24 h (Anderson and McIntosh, 1991). Here we describe the first complete segregation of a directed inactivation of *psaA* in *Synechocystis* 6803 by selection under LAHG conditions.

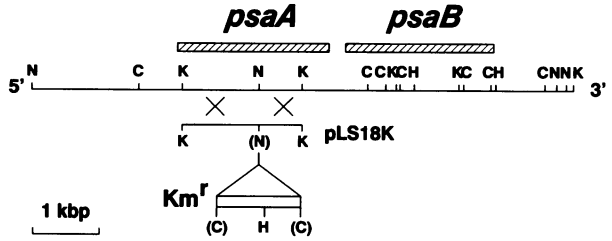

Fig. 1. Restriction map of the insert region from plasmid pLS18K and of the *psaA-psaB* operon in *Synechocystis* 6803. Protein coding regions for *psaA* and *psaB* are shown as hatched boxes. Cloned *Synechocystis* 6803 DNA in pLS18K is indicated as a line (insert from pLS18), while the Km^R gene from Tn*903* is indicated as a box. Potential regions of cross-over integration into *Synechocystis* 6803 chromosome are shown as crosses. Restriction sites are: N, *Nco*I; C, *Hinc*II; K, *Kpn*I; H, *Hin*dIII. Sites in parentheses were destroyed in the construction of pLS18K.

Results

Construct interrupting psaA

A 1.8 kbp *Kpn*I fragment, encoding most of the *psaA* gene from *Synechocystis* 6803 (Smart and McIntosh, 1991), in pUC119 (Vieira and Messing, 1987) (plasmid pLS18), was digested with *Nco*I and the resulting single-stranded DNA overhangs were digested briefly with mung bean nuclease. A 1.25 kbp *Hinc*II fragment, encoding an aminoglycoside 3′-phosphotransferase from Tn*903* conferring resistance to kanamycin (Km) (Oka *et al.*, 1981), was purified from pUC4K and ligated into the blunted *Nco*I site of pLS18. This was transformed into *Escherichia coli* DH5α (Gibco-BRL) and Km-resistant (Km^R) colonies were recovered. The resulting plasmid, pLS18K, had lost the *Nco*I site and the *Hinc*II sites flanking the Km^R cassette. A restriction map of the construct from pLS18K is shown in Figure 1.

Transformation of Synechocystis 6803 and selection conditions

Synechocystis 6803 that had been maintained under LAHG conditions for two subcultures was transformed with the plasmid pLS18K. Selection for Km^R colonies was performed under both mixotrophic (continuous light and

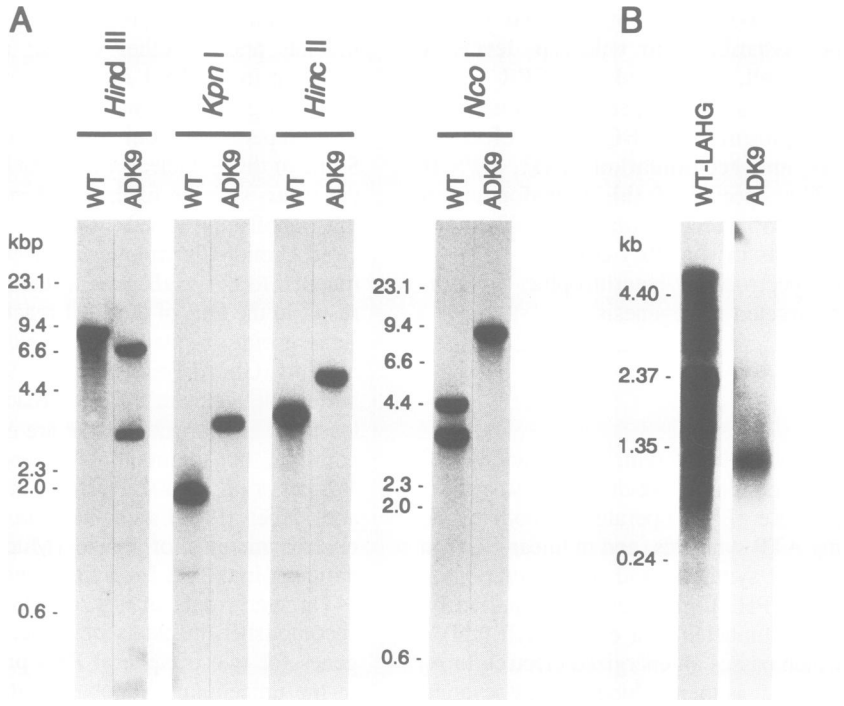

Fig. 2. (**A**) Autoradiographs of Southern blots of wild-type *Synechocystis* 6803 and ADK9 DNA probed with the insert from pLS18. Enzymes used for digestion are indicated. 7.5 μg of DNA were loaded per lane. The *Hin*dIII, *Kpn*I and *Hinc*II digests were run on the same gel; the *Nco*I digest was run on a separate gel. Size markers were from λ digested with *Hin*dIII and are indicated in kbp. (**B**) Autoradiographs of Northern blots of WT-LAHG RNA (15 μg) and ADK9 RNA (15 μg) probed with the insert from pLS18. Size markers are from an RNA ladder (Gibco-BRL) stained with ethidium bromide and are indicated in kb.

5 mM glucose) and LAHG conditions. Single Km^R colonies were streaked to at least five serial plates to obtain full segregation of the mutation. Colonies on plates required ~25 days of growth to reach adequate size. One Km^R colony recovered under LAHG conditions (ADK9) and one recovered under mixotrophic conditions (ALT6) were analyzed further.

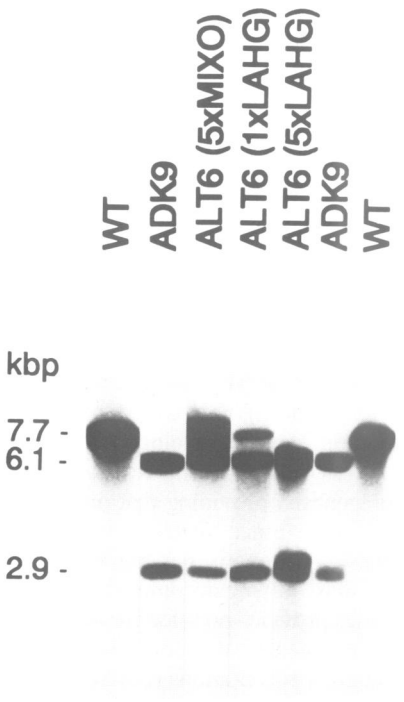

Fig. 3. Autoradiographs of Southern blots of wild-type *Synechocystis* 6803, ADK9 and ALT6 DNA digested with *Hin*dIII and probed with the insert from pLS18. 5.0 μg of DNA were loaded per lane. The four lanes on the left were run on one gel; the three lanes on the right were run on another gel. DNA was isolated from ALT6 after five serial plates in mixotrophic conditions (5 × MIXO), after five plates in mixotrophic and one plate in LAHG conditions (1 × LAHG), and after five plates in mixotrophic and five plates in LAHG (5 × LAHG). Kanamycin selection was maintained throughout. Sizes were estimated from λ digested with *Hin*dIII and are indicated in kbp.

Genetic mapping and segregation analysis

Southern blots of genomic digests from ADK9 were probed with the 1.8 kbp *Kpn*I insert from pLS18 (Figure 2A). A restriction map of the *psaA-psaB* region is shown in Figure 1. In all the digests of ADK9 DNA, no wild-type copies of the *psaA* gene were detected (Figure 2A), even after prolonged exposure (data not shown). Hybridization of *psaA* to a *Hin*dIII digest of ADK9 DNA revealed a new *Hin*dIII site (within the Km^R cassette), creating fragments of 6.1 and 2.9 kbp, rather than the wild-type 7.7 kbp fragment. The *psaA* probe hybridized to an 8.7 kbp *Nco*I fragment of ADK9 DNA, rather than to the 4.3 and 3.2 kbp wild-type fragments, confirming the loss of this site by the insertion of the Km^R cassette. Hybridization to *Hin*cII and *Kpn*I digests of ADK9 DNA showed an increase in the length of the hybridizing fragments due to the insertion of the Km^R gene. The wild-type *psaA* and *psaB* genes are arranged in tandem and are co-transcribed (Smart and McIntosh, 1991). Insertion of the Km^R cassette caused premature termination of transcription, yielding a 1.2 kb message, rather than the wild-type, 5.0 kb message (Figure 2B).

DNA was isolated from ALT6 at three stages of segregation: after five serial platings under mixotrophic conditions (5 × MIXO), after five plates under mixotrophic and one plate under LAHG conditions (1 × LAHG), and after five plates under mixotrophic and five under LAHG conditions (5 × LAHG). Km selection was maintained for all subcultures. These DNA samples were digested with *Hin*dIII, separated by electrophoresis, blotted and hybridized with the *psaA* probe described above to determine the degree of segregation of the *psaA* mutation at each stage (Figure 3). In addition, the hybridization to fragments of 7.7, 6.1 and 2.9 kbp was quantitated using a Betascope to determine the percentage of hybridization to wild-type or mutant fragments (data not shown). The DNA from ALT6 (5 × MIXO) has a high percentage of wild-type copies of *psaA* (Figure 3); quantitation showed the ratio of wild-type to mutant copies to be 3:1. After only one plate grown under LAHG conditions, the ratio of wild-type to mutant copies shifts dramatically to 1:3. Five serial plates under LAHG conditions is sufficient for complete segregation of the *psaA* mutation (Figure 3, 5 × LAHG).

Table I. Chlorophyll determination and photosynthesis assays

	WT-MIXO	WT-LAHG	ADK9
Chlorophyll determination			
Whole cells[a]			
μg chl/ml/OD_{730}	3.66	0.81	0.37
Membranes[b]			
μg chl/mg protein	43.1	9.9	5.2
Oxygen evolution			
1 mM DCBQ[c]			
μmol O_2/mg chl/h	205.7 ± 16.0	832.0 ± 193.6	1689.7 ± 306.1
μmol O_2/l/OD_{730}/h	752.9	673.9	625.2
No added acceptor[d]			
μmol O_2/mg chl/h	147.9 ± 29.5	537.0 ± 79.3	286.9 ± 79.3
μmol O_2/l/OD_{730}/h	541.3	435.0	106.2

[a]Averages of at least five samples of each cell type.
[b]Averages of two samples of each cell type.
[c]Averages of at least 14 trials for each cell type.
[d]Averages of at least six trials for each cell type.

Chlorophyll quantitation and oxygen evolution

The amount of chlorophyll in whole cells harvested at late log phase and in isolated membranes is presented in Table I for WT-MIXO, WT-LAHG and ADK9 cells. The amount of chlorophyll per wild-type cell drops slightly more than 4-fold when grown under LAHG conditions. The ADK9 mutant has less than half the chlorophyll per cell, when compared with WT-LAHG. The relative amounts of chlorophyll per mg protein in membranes from the three cell types approximately fits the same ratios as chlorophyll per cell (Table I). The oxygen evolution rates of all three cell types, assayed in the presence of DCBQ, which accepts electrons from PS II in a DCMU-sensitive reaction (Bowlby *et al.*, 1988), are approximately equal, when normalized to equal cell number (Table I). The whole chain photosynthesis rates, measured with no added acceptor, are only slightly lower for WT-LAHG compared with WT-MIXO on a per cell basis (Table I). However, the oxygen evolution rate with no artificial acceptor for ADK9 is ~4-fold lower than for WT-LAHG, when normalized to equal cell number (Table I). The number of cells per ml, relative to absorbance at 730 nm (OD_{730}), was approximately equal for all three cell types (data not shown), indicating that OD_{730} is a good estimate of cell number.

Growth characteristics

The growth of ADK9 was indistinguishable from that of wild-type cells under LAHG conditions (data not shown). However, after several attempts, ADK9 would not divide under continuous illumination of 20 μmol/m^2/s, our standard conditions for growth of wild-type *Synechocystis* 6803 (Jansson *et al.*, 1987).

EPR spectra

Spectra from membranes isolated from WT-MIXO, WT-LAHG and ADK9 cells are shown in Figure 4. The spectra from illuminated wild-type membranes show a prominent feature with a linewidth of 0.8–1.0 millitesla (mT) centered at $g = 2.0026$, characteristic of signal I from P_{700}^+ (panel A) (Norris *et al.*, 1971). However, the signal from illuminated ADK9 membranes had a 2.0 mT linewidth centered at $g = 2.0040$, typical of signal II from Y_D^+ (panels A and E) (Barry and Babcock, 1987). Signal II was detected from membranes of all three cell types after dark adaptation (panel B). In ADK9, there is no detectable signal I (panels A and E).

Detection of proteins by antibodies

Thylakoid proteins from WT-MIXO, WT-LAHG and ADK9 were separated by SDS–PAGE, transferred to nitrocellulose, and probed with antibodies raised to PS I proteins and to the D1 protein of PS II (Figure 5). Approximately equal protein was loaded in each lane, 120 μg protein representing 5 μg chl for WT-MIXO, 150 μg protein for WT-LAHG (1.5 μg chl) and 145 μg protein for ADK9 (0.75 μg chl). Antibodies raised to PSA-A and PSA-B from spinach cross-reacted to a diffuse band with apparent molecular weights of 60–65 kDa in both wild-type samples, but not in ADK9 (panel A). This anomalous behavior of the P_{700}-apoproteins in SDS–PAGE, migration at a lower molecular weight than the predicted 83 kDa, has been observed previously and is probably due to the highly hydrophobic nature of these proteins (Fish *et al.*, 1985). Antibodies raised to PSA-D cross-reacted to protein of ~15 kDa in both wild-type samples, but not in ADK9 (panel B). Similarly, antibodies raised to PSA-C cross-reacted to a protein of ~9 kDa in the wild-type samples (panel C), including a purified PS I preparation, but not in the ADK9 sample. The PSA-C antibody cross-reacted to high molecular weight background bands in wild-type and ADK9 thylakoids, but not in a PS I preparation. With all three antibodies, development was allowed to continue until background bands appeared in the ADK9 lane, to ensure there was no signal from a specific cross-reaction in that lane. The intensity of signal from WT-MIXO was greater than from WT-LAHG for all three PS I antibodies. Antibodies raised to PSB-A cross-reacted to a diffuse band of ~32 kDa in all three samples with approximately equal intensity.

Discussion

Synechocystis 6803 as a genetic system

Synechocystis 6803 serves as an excellent system for the genetic alteration of photosynthesis proteins. Its ease of transformation, active homologous recombination, and ability to express bacterial drug resistance genes simplify targeted mutagenesis, providing that proper conditions for selection are met. Since *Synechocystis* 6803 maintains multiple copies of its genome per cell, complete segregation of a mutation may only be accomplished if the presence of wild-type copies provides no selective advantage to the cell. In order to mutagenize the core polypeptides of PS I, heterotrophic growth conditions (LAHG) for *Synechocystis* 6803 were developed by Anderson and McIntosh (1991). Procedures for isolations of PS I and PS II complexes (Rögner *et al.*, 1990) and well-characterized PS I and PS II EPR signals (Barry and Babcock, 1987; Golbeck and Bryant, 1991) make analysis of mutants relatively straightforward.

Response of wild-type cells to LAHG conditions

Wild-type *Synechocystis* 6803 grown under LAHG conditions has dramatically less chlorophyll per cell, when compared with WT-MIXO. When PS II activity and EPR signal II intensity are expressed per cell, the amount of active PS II in WT-LAHG was only slightly less than in WT-MIXO. The signal intensity from PSB-A antibody was approximately equal in WT-MIXO and WT-LAHG, while the signals from PS I antibodies were lower in WT-LAHG compared with WT-MIXO. The ratio of PS I to PS II, reflected by the relative intensity of EPR signal I to signal II, was greater for WT-MIXO than for WT-LAHG; on the order of 8–10:1 for WT-MIXO and 2–3:1 for WT-LAHG (Figure 4, panels C and D). A similar ratio of PS I to PS II for light-grown cells (9:1) was determined by Rögner *et al.* (1990). Thus, the reduction in chlorophyll in WT-LAHG cells was due primarily to a reduction in the amount of PS I, while the amount of PS II remained approximately the same. However, the pool of *psaA-psaB* transcript drops only 20% when *Synechocystis* 6803 is grown under LAHG conditions, while the pool of *psbA* message drops >70% (Smart and McIntosh, 1991). This indicates that there is some form of post-transcriptional regulation of PS I and PS II accumulation in cells grown under LAHG conditions.

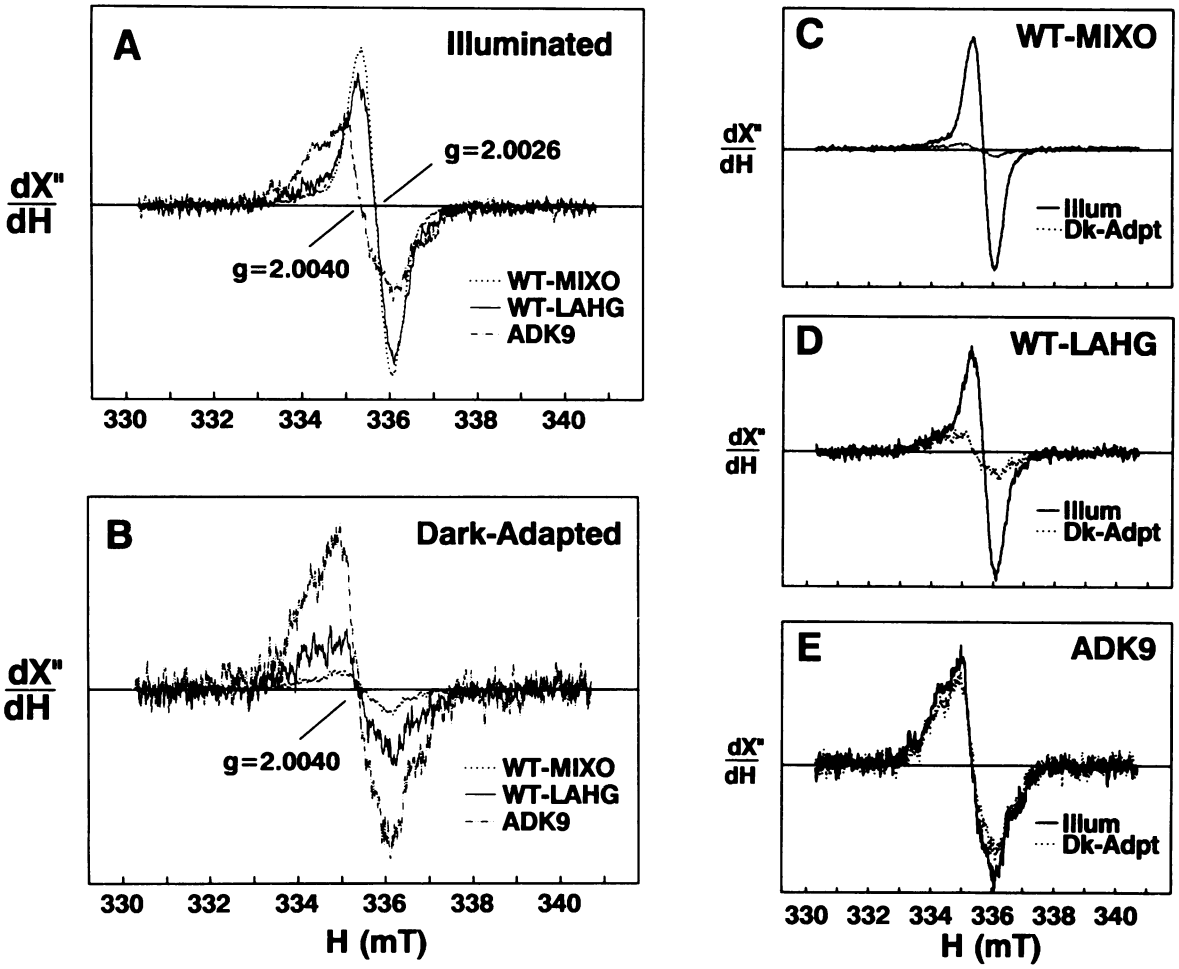

Fig. 4. EPR spectra from thylakoid membranes isolated from wild-type *Synechocystis* 6803 grown under mixotrophic (WT-MIXO) or LAHG conditions (WT-LAHG), and from ADK9. Panel **A** is spectra from all three cell types under illumination, panel **B** is spectra from all three cell types after dark adaptation. Panels **C**, **D** and **E** are the same spectra as in A and B presented as illuminated and dark-adapted for each cell type. Spectra are normalized to equal spectrometer gain and to 1 mg chl/ml.

Inactivation of psaA

We have used LAHG conditions for the selection and complete segregation of a *psaA*-inactivation mutant of *Synechocystis* 6803. This mutant, ADK9, contains no detectable functional PS I. Signal I, from P_{700}^+, was not present in the EPR spectrum of ADK9. Antibodies raised to the P_{700}-apoproteins from spinach, which cross-reacted to protein from wild-type *Synechocystis* 6803, did not detect the P_{700}-apoproteins in ADK9 thylakoids. Upon probing a Northern blot of ADK9 RNA with the *psaA* gene, only a truncated *psaA-psaB* transcript ~1.2 kb in length was detected (5.0 kb in wild-type, Figure 2B). This is too short to encode the full length of PSA-A and none of PSA-B. ADK9 displayed greatly reduced rates of oxygen evolution with no artificial acceptor. The low rate of oxygen evolution that was observed in whole ADK9 cells may simply represent saturation of the quinone pool or it may indicate there was an undefined electron acceptor from PS II. This activity was fully inhibited by DCMU, an inhibitor of PS II (data not shown). Three possible electron acceptors for PS II in ADK9 are: ferredoxin, which reduces $NADP^+$, as proposed by Arnon *et al.* (1981); cytochrome oxidase, via the cytochrome b_6–f complex, which is shared between photosynthesis and respiration in cyanobacteria (Sandmann *et al.*, 1984); or a hydrogenase, which would reduce H^+ to hydrogen gas and has been purified from cyanobacteria (Ewert and Smith, 1989). The amount of chlorophyll per cell was greatly reduced in ADK9, allowing the turquoise blue color of the phycobilisomes to predominate. This would be expected with the loss of the PS I core polypeptides, which bind over 100 chlorophyll molecules per P_{700} (Lundell *et al.*, 1985). By inspection of cells on plates illuminated with long-wave UV light (366 nm), fluorescence yield from ADK9 was greater than from WT-LAHG or WT-MIXO, a classic phenotype of mutants impaired in photosynthesis (Miles *et al.*, 1979; Girard *et al.*, 1980).

Antibodies raised to PSA-C and to PSA-D, membrane-extrinsic proteins of PS I, which cross-reacted to protein in wild-type thylakoids, failed to detect those proteins in ADK9 thylakoids. This suggest that PSA-C and PSA-D do not accumulate in the absence of P_{700}-apoproteins. Despite the absence of the PS I core in ADK9, PS II assembled and formed functional complexes. The EPR spectrum from ADK9 included signal II from Y_D^+, a tyrosine radical in the D2 polypeptide. Adding DCBQ, an acceptor of PS II, stimulated oxygen evolution to near wild-type rates when expressed on a per cell basis. The D1 polypeptide accumulated in ADK9, since antibodies raised to D1 from *Amaranthus hybridus* cross-reacted to protein of the expected molecular weight (32 kDa) in ADK9 thylakoids. The loss

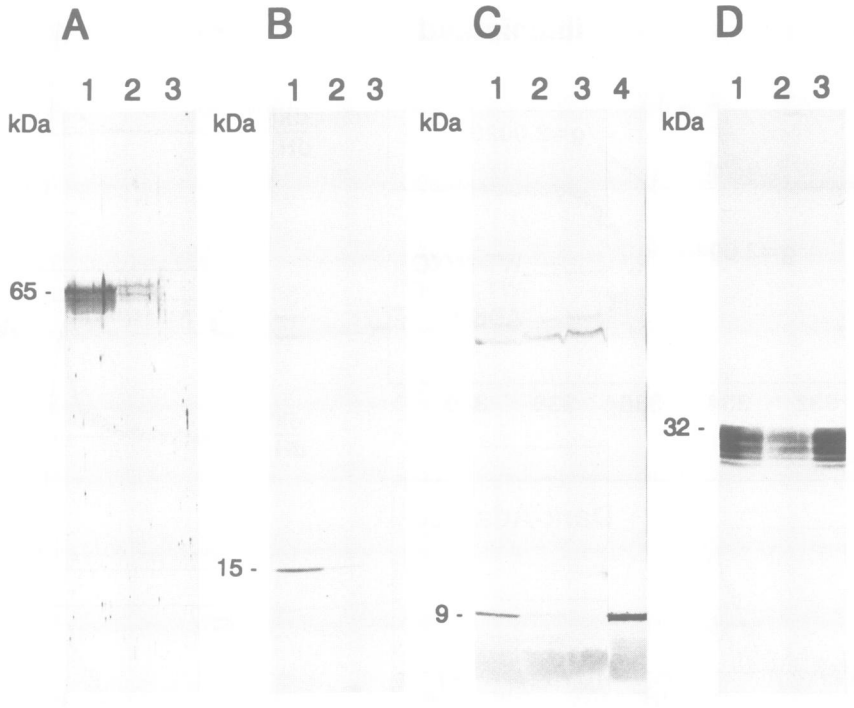

Fig. 5. Immunoblots of thylakoid proteins from WT-MIXO, WT-LAHG and ADK9. Lane 1 is WT-MIXO (5 μg chl); lane 2 is WT-LAHG (1.5 μg chl); lane 3 is ADK9 (0.75 μg chl); lane 4 in panel **C** is purified PS I complex from wild-type *Synechocystis* 6803 (5 μg chl). Immunoblots were probed with PSA-A/PSA-B antibody (panel **A**), PSA-D antibody (panel **B**), PSA-C antibody (panel **C**) and PSB-A antibody (panel **D**). Relative molecular weights were estimated by comparison with prestained size standards and are expressed in kDa.

of small PS I proteins concomitant with the inactivation of the PS I core, with the continued assembly of functional PS II, corresponds to previously characterized nuclear (Chua *et al.*, 1975; Girard *et al.*, 1980) and plastome (Girard-Bascou *et al.*, 1987) mutants of *C.reinhardtii* and a nuclear mutant of barley (Møller *et al.*, 1980). Genetic inactivation of PS I in strains with site-directed mutations in PS II may facilitate their analysis by removing contaminating signals from PS I.

Although ADK9 grew at wild-type rates under LAHG conditions, it did not divide under continuous illumination of 20 μmol/m^2/s. The loss of PS I and the chlorophyll bound to it probably made ADK9 extremely sensitive to photoinhibition. We have not determined the maximum irradiance under which ADK9 will grow. Since ADK9 did evolve oxygen, although at a much lower rate, these cells may be able to grow autotrophically (in the light without glucose) by oxidizing water and passing those electrons to an as yet undefined acceptor.

Conclusions

The use of LAHG conditions is sufficient for complete segregation of mutations in the PS I core in *Synechocystis* 6803. These conditions eliminate the selective advantage conferred by the presence of wild-type PS I and avert the lethal effect of continuous light. The success of this inactivation of *psaA* is encouraging for planned site-directed mutagenesis in the study of structure–function relationships in PS I.

Materials and methods

Materials

All chemicals and reagents were of the highest purity available and were obtained from Sigma (St Louis, MO) or Research Organics (Cleveland, OH). Enzymes were obtained from Gibco-BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). Radioactive nucleotide ([α-^{32}P]dATP) was obtained from Amersham (Arlington Heights, IL).

Strain and growth conditions

A glucose-tolerant (Williams, 1988), dark-growth-adapted (Anderson and McIntosh, 1991) strain of *Synechocystis* sp. PCC 6803 was grown in BG-11 medium supplemented with 5 mM *N*-tris[hydroxymethyl]-2-aminoethane-sulfonic acid (TES), pH 8.0, and 5 mM glucose, as previously described (Jansson *et al.*, 1987). Mixotrophic cultures (WT-MIXO) were grown in continuous light (20 μmol/m^2/s), while heterotrophic cultures (WT-LAHG and ADK9) were grown under LAHG conditions [complete darkness, except 5 min of light (40 μmol/m^2/s) every 24 h] (Anderson and McIntosh, 1991). For growth on solid medium, the above medium was supplemented with 1.5% w/v agar and 0.3% sodium thiosulfate as described (Anderson and McIntosh, 1991). When appropriate, media were supplemented with 5 μg/ml Km sulfate. Cells used for membrane isolation were grown in 10–15 l of medium and were harvested using a Sorvall continuous flow rotor. Cell growth was measured by absorbance at 730 nm (OD_{730}) in a Gilford Response spectrophotometer (Oberlin, OH) using a glass, 10 mm pathlength cuvette. Cells were counted in a hemacytometer. Chlorophyll determinations were performed by methanol extraction using the extinction coefficients of Lichtenthaler (1987).

Nucleic acid manipulations

All nucleic acid manipulations were performed using standard techniques (Sambrook *et al.*, 1989), except where otherwise noted. Genomic DNA was isolated from *Synechocystis* 6803 essentially as described (Williams, 1988), fractionated by agarose gel electrophoresis, and transferred to nitrocellulose (Southern, 1975). RNA isolation and conditions for hybridizations were previously described (Smart and McIntosh, 1991). A 1.8 kbp *Kpn*I fragment encoding most of *psaA* from *Synechocystis* 6803 (pLS18) (Smart and McIntosh, 1991) was random primer labeled (Feinberg and Vogelstein, 1983) and used as a probe. Quantitation of hybridization to Southern blots was performed using a Betascope (Betagen, Waltham, MA). Transformation of *Synechocystis* 6803 was performed essentially as described (Williams, 1988), except that cells were transferred to selective medium 30 and 60 h after initial plating on non-selective medium.

Oxygen evolution assays

Rates of oxygen evolution were measured using whole cells as previously described (Debus *et al.*, 1988). For wild-type cells, 10 μg chl/ml was used;

for ADK9 cells, 5 μg chl/ml was used. Light (from projector lamps) passed through a copper sulfate solution and filtered with red cellophane provided saturating illumination. DCBQ (1 mM) was added where appropriate. Cells for oxygen evolution assays were grown to late log phase (OD_{730} = 0.6–1.0), harvested, and washed twice with BG-11.

Thylakoid membrane isolation

Cells for thylakoid membrane isolation were grown to late log phase, harvested by centrifugation, washed with BG-11, and resuspended in break buffer [0.8 M sucrose, 20 mM 2-[*N*-morpholino]-ethansulfonic acid (MES), pH 6.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mg/ml DNase I; 3 mg/l phenylmethylsulfonyl fluoride (PMSF); 0.6 mg/l pepstatin a; 1.8 mg/l *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)]. The cells were broken in a Bead Beater (Biospec Products, Bartlesville, OK) essentially as described by Rögner *et al.* (1990). Unbroken cells and cell debris were pelleted at 6000 *g* for 15 min (Sorvall GSA rotor). The membranes were then precipitated with 25 mM calcium chloride and 5% w/v polyethylene glycol mol. wt 8000 and pelleted at 27 000 *g* for 15 min (Sorvall GSA rotor). The membranes were resuspended in freeze buffer (25% v/v glycerol, 20 mM calcium chloride, 20 mM MES, pH 6.0), pelleted at 48 000 *g* for 30 min (Sorvall SS-34 rotor), then resuspended in freeze buffer and stored at −70°C. Purified PS I complex from wild-type *Synechocystis* 6803 was provided by J.Golbeck (Department of Biochemistry, University of Nebraska, Lincoln, NE). Protein concentration was determined using the Lowry assay (Lowry *et al.*, 1951).

EPR spectra

Isolated thylakoid membranes were pelleted and resuspended to a final concentration of 0.28–1.7 mg chl/ml in BG-11–16 μM DCMU–0.25 mM EDTA. EPR spectra were obtained as previously described (Barry and Babcock, 1987). Illumination was provided by a high-intensity microscope light. Dark-adaptation was for at least 10 min. Conditions were as follows: power, 2 mW; field modulation, 4 G; time constant, 200 ms; sweep time, 100 s. Spectra were averaged from at least five scans. The gain used for dark-adapted samples and for illuminated ADK9 was 8×10^6. The gain used for illuminated WT-MIXO was 1×10^6 and for illuminated WT-LAHG was 2×10^6

SDS – PAGE and immunoblotting

Protein samples were separated by electrophoresis on a 10–17.5% gradient acrylamide resolving gel with a 5% stacking gel (Laemmli, 1970). Samples were prepared as described by Wynn *et al.* (1989). Gels were run at 25 mA constant current for 7–8 h. Protein was visualized using Coomassie stain or transferred to nitrocellulose overnight at 1 mA with cooling (Towbin *et al.*, 1979). Relative molecular weights were estimated by comparison with prestained molecular size standards (Diversified Biotech, Newton Center, MA). Immunoblots were incubated for at least 1 h in blocking solution [10 mM Tris, pH 7.4, 0.9% NaCl, 1% bovine serum albumin (BSA), 0.02% sodium azide (NaN_3), 2% dry milk], then incubated with the primary antibody (1:500 dilution) for 1–1.5 h at room temperature. After washing off the primary antibody (three times for 15 min with 10 mM Tris, pH 7.4; 0.9% NaCl; 0.02% NaN_3; 0.1% Triton X-100; 0.05% SDS; 0.1% BSA) the blots were incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) (1:3000 dilution of a 0.5 mg/ml solution in block buffer). The detection reaction was performed in development buffer (100 mM Tris, pH 8.8; 100 mM NaCl; 5 mM magnesium chloride) using BCIP (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) and NBT (nitro-blue tetrazolium) as substrates (Knecht and Dimond, 1984). Antibodies raised in rabbit against the P_{700}-apoproteins (PSA-A and PSA-B) from spinach were the kind gift of Nathan Nelson (Roche Institute, Nutley, NJ). Antibodies raised to the PSA-C protein from *Synechococcus* (Henry *et al.*, 1990) were the gift of James Guikema (Division of Biology, Kansas State University, Manhattan, KS). Antibodies raised to the 32 kDa D1 polypeptide from *A.hybridus* were previously described (Ohad *et al.*, 1985). PSA-D antibodies, raised in rabbit to spinach protein, were the gift of Richard Malkin (Division of Molecular Plant Biology, University of California, Berkeley, CA).

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Since submission of this manuscript, directed mutagenesis of the chloroplast gene *psaC* in the alga *Chlamydomonas reinhardtii* has been reported (Takahashi,Y., Goldschmidt-Clermont,M., Soen,S.-Y., Franzén,L.G. and Rochaix,J.-D. (1991) *EMBO J.*, **10**, 2033–2040.