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Requirement for the H Phosphoprotein in Photosystem II of *Chlamydomonas reinhardtii*[1]

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To dissect the expression of the *psb*B gene cluster of the *Chlamydomonas reinhardtii* chloroplast genome and to assess the role of the photosystem II H-phosphoprotein (PSII-H) in the biogenesis and/or stabilization of PSII, an *aad*A gene cassette conferring spectinomycin resistance was employed for mutagenesis. Disruption of the gene cluster has no effect on the abundance of transcripts of the upstream *psb*B/T locus. Likewise, interruption of *psb*B/T and *psb*H with a strong transcriptional terminator from the *rbc*L gene does not influence transcript accumulation. Thus, *psb*B/T and *psb*H may be independently transcribed, and the latter gene seems to have its own promoter in *C. reinhardtii*. In the absence of PSII-H, translation and thylakoid insertion of chloroplast PSII core proteins is unaffected, but PSII proteins do not accumulate. Because the deletion mutant also exhibits PSII deficiency when dark-grown, the effect is unrelated to photoinhibition. Turnover of proteins B and C of PSII and the polypeptides PSII protein A and PSII protein D is faster than in wild-type cells but is much slower than that observed in other PSII-deficient mutants of *C. reinhardtii*, suggesting a peripheral location of PSII-H in PSII. The role of PSII-H on PSII assembly was examined by sucrose gradient fractionation of pulse-labeled thylakoids; the accumulation of high-molecular-weight forms of PSII is severely impaired in the *psb*H deletion mutant. Thus, a primary role of PSII-H may be to facilitate PSII assembly/stability through dimerization. PSII-H phosphorylation, which possibly occurs at two sites, may also be germane to its role in regulating PSII structure, stabilization, or activity.

The biogenesis and structural integrity of PSII in plants and green algae requires most of the subunits of this complex. This has been demonstrated most clearly in nuclear mutants of *Chlamydomonas reinhardtii* with lesions in regulation of gene expression, as well as mutations that disrupt chloroplast structural genes (Mayfield et al., 1995). Especially at excessive light intensities, maintenance of the PSII complex is thought to be dependent upon high rates of synthesis of the D1 polypeptide in a manner that is coordinated with its rapid turnover (Barber, 1995). Recently, it has been proposed that phosphorylation of certain subunits of the PSII complex participates in activation of the proteolytic pathway (Giardi et al., 1994; Rintamaki et al., 1995a). However, phosphorylation of thylakoid proteins is best documented to promote state transitions through dissociation of light-harvesting complexes from PSII under conditions when excitation of PSI is limiting and the Cyt b_6/f complexes are in a reduced state (Bennett, 1991). At least four subunits of PSII are also usually phosphorylated in a light-dependent manner (Bennett, 1991). These proteins include D1 and D2 of the reaction center, PSII-C (the core antenna chlorophyll-protein complex, CP43), and a 9-kD protein, respectively, encoded by the chloroplast *psb*A, *psb*D, *psb*C, and *psb*H genes. Phosphorylation of D1 has not been detected in *C. reinhardtii* or bryophytes (Ikeuchi et al., 1987a; Rintamaki et al., 1995b).

It is not clear whether phosphorylation of PSII core proteins corresponds to part of the mechanism for D1 turnover, initiation of state transitions, inactivation of PSII, or a combination of such events. There are similar sequence motifs for Thr phosphorylation of the light-harvesting proteins and PSII proteins (Mullet, 1983; Michel and Bennett, 1987; Dedner et al., 1988; Michel et al., 1988), but the identity of the enzyme(s) responsible for these modifications has not been established (Hind et al., 1995; Race et al., 1995; Sokolenko et al., 1995). For cyanobacterial PSII, only the PSII-H polypeptide has been reported to undergo phosphorylation (Race and Gounaris, 1993), even though there is no corresponding consensus sequence at the N termini of the proteins from these species (Abdel-Mawgood and Dilley, 1990; Mayes and Barber, 1991). Nonetheless, universal phosphorylation of the PSII-H polypeptide would seem to reflect a fundamental physiological process: PSII-H was among the first chloroplast proteins shown to be phosphorylated and to such a high degree that it seems to undergo continual phosphorylation/dephosphorylation in the light (Bennett, 1977).

As a step toward further resolution of the requirements for small proteins in PSII biogenesis and function, we have examined the role of the PSII-H polypeptide in *C. reinhardtii*. Here, we show that deletion of the *psb*H gene leads to loss of proteins of the PSII core complex, but that turnover of these subunits is quite slow relative to that resulting from mutations affecting other subunits of the complex. We also demonstrate that formation of PSII complexes is impaired in the absence of PSII-H. This is in contrast to the situation in cyanobacteria, in which elimination of PSII-H does not affect assembly and stability of PSII, even though there is partial loss of electron transfer efficiency from the

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Abbreviations: ACA, aminocaproic acid; BAM, benzamidine; D1, PSII protein A; D2, PSII protein D; PSII-B, PSII-C, and PSII-H, PSII proteins B, C, and H; TAP, Tris-acetate phosphate.

Q_A to Q_B quinone sites (Mayes et al., 1993). Our work entailing disruption of the *psb*H gene by replacement of this sequence with that for a gene for antibiotic resistance also contributes to an understanding of the expression of an evolutionarily conserved cluster of chloroplast genes for several PSII proteins. Evidence is provided for the expression of upstream *psb*B and *psb*T genes in a manner that is independent of *psb*H transcription. Thus, the formation of a *psb*B-*psb*T-*psb*H mRNA precursor is not requisite for the generation of the mature transcripts. Moreover, interruption of the *psb*B gene cluster with an antibiotic-resistance cassette, in either a forward or reverse transcriptional orientation, has no effect on the transcription of *psb*H. Thus in *Chlamydomonas*, it appears that this gene possesses its own promoter.

MATERIALS AND METHODS

Strains and Media

Chlamydomonas reinhardtii strain 137 (mating type +) was used for all experiments. Cells were grown in TAP or in minimal media in liquid or 1.3% agar. As required, plates and liquid media were supplemented with ampicillin (100 μg mL^{-1}) or spectinomycin (100 μg mL^{-1}).

DNA Constructs

Plasmid recombination into the *psb*B/T-N-H gene cluster of the *C. reinhardtii* chloroplast genome is illustrated in Figure 1. R19, a large ctDNA *Eco*RI fragment, was the source for 51–3, a *Eco*RI/*Pst*I subclone in pBluescript SK(−). The 3.6-kb insert of 51–3 contains *psb*N, *psb*H, and part of the *trn*E coding region (Johnson and Schmidt, 1993). To confer spectinomycin and streptomycin resistance to the constructs, the 1.9-kb *aad*A cassette was utilized (Goldschmidt-Clermont, 1991). The cassette, originating from pUC-*atp*X-AAD, contains *aad*A behind the promoter and a 5′ untranslated sequence of *atp*A and is followed by the 3′ termination sequence of the *C. reinhardtii* chloroplast *rbc*L gene. The cassette was excised with *Eco*RI/*Not*I, blunt-ended with the Klenow fragment, and cloned into the *Eco*RV site of pBluescript KS(−) to produce the plasmid pBSaadA. Plasmid p(aadA)iNH was constructed by ligating the *Sma*I/*Eco*RV-excised *aad*A sequence into the blunt-ended *Bsm*I site (position 2265) of 51–3. Plasmid p(aadA)ΔH contains the *aad*A cassette but lacks the entire *psb*H gene. To create p(aadA)ΔH, the 5′ chloroplast sequences and the entire *aad*A cassette was excised from p(aadA)iNH by digestion with *Sma*I and partial digestion with *Hin*dIII, filling in the ends using the Klenow fragment and ligating into a *Nhe*I/*Eco*RI subclone of 51–3. Plasmid p(aadA)UN was created by ligating the *aad*A cassette into the *Hpa*I site at position 1394 of 51–3, whereas p(aadA)iHE was produced by insertion at the *Msc*I site at position 3404 of 51–3.

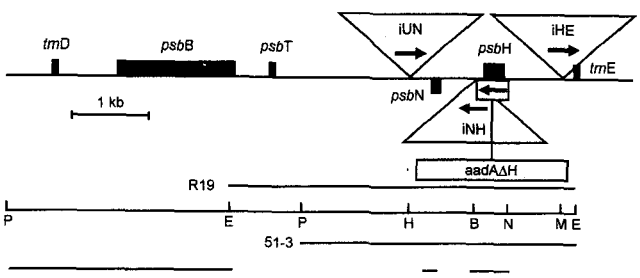

Figure 1. Mutation sites in the *psb*B/T, *psb*N, and *psb*H gene cluster. Drawn to scale are maps of the *C. reinhardtii* chloroplast genome and sites for insertional and deletion mutagenesis with the *aad*A cassette. The limits of the R19 *Eco*RI fragment and the 51–3 subfragment are also indicated. Also shown are restriction sites for *Pst*I (P), *Eco*RI (E), *Hin*dIII (H), Bsm I (B), *Nhe*I (N), and Msc I (M). The bars at the bottom of the figure indicate the respective positions of subclones used for generating the probes for the northern analyses of *psb*B, *psb*N, and *psb*H in Figure 3.

All plasmids were transformed by heat shock into XLI-Blue *Escherichia coli* competent cells and selected on Luria broth plates containing ampicillin and spectinomycin (100 μg mL^{-1}, each). Gene orientations were confirmed by restriction mapping and partial sequencing (Sequenase version 2.0, United States Biochemical). Plasmid DNA for cloning, sequencing, and transformation was isolated by standard procedures.

Algal Transformation

Transformation of *C. reinhardtii* chloroplasts with a particle gun transformation system (Biolistic PDS-1000/He, Bio-Rad) was essentially as described by Boynton and Gillham (1993). Five micrograms of plasmid DNA was ethanol-precipitated onto 12 mg of tungsten M-17 beads (Bio-Rad) and resuspended in 40 μL of 100% ethanol, and 5 μL was spread on carrier discs for each transformation event. Cells were grown to 10^6 cells mL^{-1} in TAP plus 0.5 mM 5-fluorodeoxyuridine (Sigma) to reduce ctDNA levels, and resuspended to a density of 10^8 cells mL^{-1}. Aliquots of 0.5 mL were added to 0.5 mL of molten 0.2% agar cooled to 42°C, poured in the middle of a TAP/ampicillin plate, and then placed in darkness at room temperature for 2 h. Following bombardment using 1100-psi rupture discs, the plates were incubated in darkness for 1 h before the surface agar was loosened with 4 mL of minimal medium for replating on TAP plates containing 100 μg mL^{-1} spectinomycin. After 2 weeks, colonies were restreaked on fresh antibiotic-containing plates and then individual colonies were selected for growth in liquid TAP-spectinomycin media for further analyses. When the loss of photosynthetic competence and high chlorophyll fluorescence became clear for heteroplasmic isolates of aadAΔH mutants, selection for metronidazole resistance was used to isolate homoplasmic mutants (Schmidt et al., 1977). The plating/selection protocol was repeated until homoplasmicity for the chloroplast gene disruption, as determined by Southern-blot analysis, was obtained.

Nucleic Acid Purification and Analyses

DNA for Southern-blot analyses and PCR was isolated from algal cells by the protocol described in Harris (1989), except the aqueous phase of phenol/chloroform extracts

was adjusted to 0.7 M NaCl before the addition of 0.1 volume of 10% cetyltrimethyl ammonium bromide in 0.7 M NaCl. After extraction of the solution with 1 volume of chloroform, the DNA was isopropanol-precipitated, resuspended in H_2O, and used for Southern-blot analysis. DNA for use in PCR reactions was further purified using DNA purification resin (Wizard Maxipreps, Promega) following the manufacturer's recommendation for plasmid DNA. RNA was purified by lysis of harvested cells with SDS in the presence of proteinase K, followed by chloroform/phenol extraction and then isopropanol, ethanol, and lithium chloride precipitation, as described previously (Johnson and Schmidt, 1993). The RNA (5 μg per lane) was separated on denaturing formaldehyde-containing agarose gels. Northern blotting to nylon membranes and hybridization with probes of the indicated chloroplast gene sequences were performed as previously described (Johnson and Schmidt, 1993). Probes for northern and Southern analysis were made by nick translation (Promega) of plasmid DNA in the presence of [α-^{32}P]ATP and used at 10^6 cpm mL^{-1} of hybridization buffer. The clones used for probes of *psb*A, *psb*D, and *psb*C transcripts have been described previously (Jensen et al., 1986). The clones used as probes of *psb*B/T, *psb*N, and *psb*H are indicated in Figure 1.

Determination of the *aad*A Cassette Integration Site

The presence of the *aad*A cassette in the proper chloroplast genomic fragment was demonstrated by Southern-blot analysis. Five micrograms of total DNA was digested to completion with *Eco*RI, separated on 0.7% agarose gel, and transferred to a nylon membrane following standard protocols. The membrane was probed with nick-translated 51–3, stripped, and reprobed with pBSaadA. More precise determination of the insertion site of the *aad*A cassette was further accomplished by PCR amplification of the respective flanking sequences. PCR reactions were done following standard protocols. DNA (0.1 μg) was amplified in a reaction containing 1 mM of each primer, 3 mM $MgCl_2$, buffer (Perkin-Elmer), 1 mM dNTP, and 1 μL of TAQ (Perkin-Elmer) for 40 cycles at denature, anneal, and extend temperatures of 92, 40, and 72°C, and cycle times of 1, 2, and 2 min, respectively. To PCR amplify sequences encompassing the 3′ integration sites, the primer sequences were 5′-GATGACGCCAACTACCTC, corresponding to positions 448–431 of *aad*A (as given in GenBank accession no. X02340), and 5′-CCCGCCACTGTCATC, corresponding to positions 2855–2841 of 51–3 (as given in GenBank accession no. L13303). The resulting products were 1.27 and 0.8 kb, respectively, for DNA from mutants generated from constructs p(aadA)iNH and p(aadA)ΔH. The 0.8-kb PCR product was sequenced directly using the above 51–3 primer and Sequenase (version 2.0, United States Biochemical). For the 5′ integration sites, a primer with the sequence 5′-CAGTTGGAAGAATTTGTCC of *aad*A (positions 1131–1148 of GenBank accession no. X02340) and 5′-CTGCCTAGGCAAGTAAAC of 51–3 (positions 1100–1118 of GenBank accession no. L13303) was used. DNA from both mutants yielded 1.7-kb products, which were then cloned into pBluescript SK(−) and digested with *Eco*RI and *Sma*I by filling in the ends with the Klenow fragment. Resulting clones were sequenced with T3 primer.

Fluorescence Measurements, Oxygen Evolution, and Growth Curves

Fluorescence was measured at 27°C with cells in liquid cultures using a pulse-amplitude-modulation fluorometer (PAM 101, 103, Walz, Effeltrich, Germany). Cells were first dark-adapted for 5 min. Initial fluorescence was measured with weak (<1 μmol photons m^{-2} s^{-1}) pulsed red light. For maximum fluorescence measurements, cells were exposed to flashes of saturating white light (5000 μmol photons m^{-2} s^{-1}) of 100-ms duration. The light source was the Walz KL1500 module with a FL103 filter. Oxygen evolution and consumption were assayed with an oxygen electrode (Rank Brothers, Cambridge, UK) with cells grown in liquid culture. Growth rates were determined by hemocytometer counting.

Pulse-Chase Labeling

For radioactive labeling, log phase cells were harvested by centrifugation (2000*g*, 3 min) and suspended at a chlorophyll concentration of 0.1 mg mL^{-1} in TAP medium without sulfate. The cells were incubated for 25 min at room temperature under a photon flux density of 100 μmol photons m^{-2} s^{-1} prior to cycloheximide addition (10 μg mL^{-1} final concentration). After 5 min, carrier-free $H_2{}^{35}SO_4$ (ICN) was added to 0.5 mCi mL^{-1} cell suspension. Pulse labeling was terminated by the addition of 1 M Na_2SO_4 (50 mM final concentration). Labeling periods were 10 min for subsequent chases (of 2- and 4-h duration, respectively) and 20 min for samples used for Suc density gradient separation.

Thylakoid Isolation and Density Gradient Fractionation

For polypeptide pattern and immunoblot analyses thylakoids were isolated by flotation in Suc step gradients, whereas membranes of pulse-chase-labeled cells were obtained by NaOH extraction (Jensen et al., 1986). For density gradient fractionation of thylakoid complexes, radiolabeled cells were collected by centrifugation (2,000*g* for 3 min) and resuspended to a chlorophyll concentration of 500 μg mL^{-1} in 0.3 mL of 0.3 M Suc, 5 mM Hepes/NaOH (pH 7.5), 1 mM $MgCl_2$, 5 mM ACA, 1 mM BAM, and 50 mM Na_2SO_4. Cells were broken with 80 bursts of sonication with a sonifier microtip. Starch was removed by centrifugation at 2,000*g* for 3 min and thylakoids in the supernatant were pelleted (48,000*g*, 20 min). Thylakoids were resuspended in 5 mM Hepes/NaOH (pH 7.5), 10 mM Na-EDTA, 5 mM ACA, and 1 mM BAM, and were centrifuged again at 48,000*g*. The sedimented membranes were solubilized for 60 min at 4°C in 1% *n*-dodecyl-β-D-maltoside, 5 mM ACA, and 1 mM BAM to yield a detergent-to-chlorophyll ratio of 10:1. Suc density gradients were prepared by a freeze-thaw method (Bassi and Simpson, 1987). In each ultracentrifuge tube, 10.8 mL of 0.5 M Suc, 10 mM Hepes/NaOH (pH 7.6), 0.03% *n*-dodecyl-β-D-maltoside, 5 mM ACA, and 1 mM BAM were frozen and thawed at 4°C. The upper 0.8 mL of the gradi-

ents was withdrawn before loading 90-μg samples of chlorophyll. Gradients were spun for 17 h at 270,000*g* and 1-mL fractions were harvested from top to bottom of the tubes. A small pellet was collected individually. To concentrate proteins, 50 μL of a homemade wheat germ extract was added to all fractions and then TCA-precipitated. Pellets were dissolved in equal volumes of gel sample buffer and either used for gel electrophoresis or for scintillation counting of the incorporated ^{35}S.

Gel Electrophoresis and Immunoblotting

Polyacrylamide gels were as described in Jensen et al. (1986). Samples for electrophoresis were suspended in 60 mM Tris/HCl (pH 8.5), 2% lithium dodecyl sulfate, 60 mM DTT, 5 mM ACA, 1 mM BAM, and 12% Suc. For TCA precipitates, the buffer contained 180 mM Tris base instead of Tris/HCl. Prior to loading, samples were boiled for 1 min. Gels were loaded on the basis of chlorophyll amount (Coomassie blue-stained gels, immunoblots), fraction volume (density gradients), or equal amounts of ^{35}S incorporated into proteins (pulse-chase samples), as determined by the Mans and Novelli (1961) procedure.

Thylakoid membrane proteins were visualized by staining with 0.5% Coomassie blue R. Incorporation of ^{35}S into the proteins was visualized by fluorography. For immunodetection, proteins were transferred to nitrocellulose membranes and incubated with antisera provided by Dr. M. Ikeuchi (University of Tokyo) to spinach D1 or D2 proteins. Immunodetection with alkaline-phosphatase-conjugated secondary antibody, 5-bromo-4-chloro-3-indolyl phosphate, and *p*-nitroblue tetrazolium was performed following standard washing and blocking procedures as modified by Evrard et al. (1990).

RESULTS

Mutant Construction

Previous studies have shown that the region of the chloroplast genome of *C. reinhardtii* harboring *psb*B, the gene encoding the PSII-B protein of the proximal CP47 light-harvesting complex, resembles that of vascular plants in that genes encoding three other PSII proteins are located downstream (Johnson and Schmidt, 1993; Monod et al., 1994). Hence, *psb*B is immediately followed by *psb*T, and *psb*N (on the opposite DNA strand) and *psb*H are further downstream. Unlike vascular plants, the *pet*B and *pet*D genes for Cyt b_6 and subunit IV of the Cyt b_6/f complex are located elsewhere on the *C. reinhardtii* chloroplast genome (Buschlen et al., 1991). It is well established that the *psb*B locus in plants represents an operon, the transcription of which results in the accumulation of a variety of pre-mRNAs, some of which undergo translation without completion of maturation (Barkan, 1988). On the basis of nuclear mutations that impair accumulation of the *psb*B-*psb*T and *psb*H transcripts (Sieburth et al., 1991; Monod et al., 1992; Johnson and Schmidt, 1993), it has been assumed that this gene cluster is also polycistronic in *C. reinhardtii* even though the intergenic regions are rather large (approximately 2000 bp). On the other hand, the multiple *psb*H transcripts that accumulate in wild-type *C. reinhardtii* could originate from transcriptional initiation from an upstream promoter sequence (Johnson and Schmidt, 1993). Unfortunately, in vitro capping experiments have failed to identify a primary transcript for any unit of the *psb*B gene cluster (Monod et al., 1992; Johnson and Schmidt, 1993).

To resolve the mode of expression of the *psb*H gene and to elucidate the functions of the PSII-H protein in chloroplasts, we constructed a series of insertion and deletion mutants of the *psb*B-*psb*T-*psb*N-*psb*H gene cluster of *C. reinhardtii*. As illustrated in Figure 1 and described in "Materials and Methods," this was achieved by constructing plasmids harboring the *E. coli aad*A gene expression cassette described by Goldschmidt-Clermont (1991) with appropriate surrounding sequences of the chloroplast genome. Following several rounds of selection for spectinomycin resistance conferred by aminoglycoside 3″-adenyltransferase, several homoplasmic strains were obtained in which the *aad*A cassette interrupted the chloroplast genome between *psb*T and *psb*N (mutant iUN), between *psb*N and *psb*H (mutant iNH), and between *psb*H and *trn*E (mutant iHE). In addition, aadAΔH mutants in which the *psb*H coding sequence and its 5′ and 3′ flanking sequences were replaced by the *aad*A cassette were isolated from colonies exhibiting high chlorophyll fluorescence on spectinomycin-containing medium. The absence of photosynthetic electron transport activity in cultures of aadAΔH isolates derived from individual colonies was apparent from their metronidazole resistance, an initial-fluorescence to maximum-fluorescence ratio of 1, the absence of photosynthetic oxygen evolution, and failure to grow photoautotrophically (not shown). The other mutants exhibited wild-type phenotypes for each of these parameters for photosynthetic competence.

DNA Analyses

After transformants were obtained, the location of the cassettes in chloroplast genome fragment R19 was confirmed by Southern analysis, as illustrated in Figure 2. Southern blotting of *Eco*RI digested total algal DNA and probing with the cassette construct pBSaadA (Fig. 2A) revealed hybridization to a novel 3.1-kb band in the iNH mutant (Fig. 2A, lane 1) and a 2.7-kb band in the aadAΔH mutant (Fig. 2A, lane 2), as expected. In addition, pBSaadA also hybridizes to a 6-kb band in both wild-type and mutant algae due to the *atp*A and *rbc*L sequences in the plasmid (Fig. 2A, lanes 1, 2, and 4). Hybridization of the same blot with clone 51–3 (Fig. 2B) reveals the original R19 *Eco*RI band of 4.4 kb in wild-type cells (Fig. 2B, lane 4). The presence of a new *Eco*RI site in the *aad*A construct results in the loss of this band and its replacement with 3.2- and 3.1-kb bands in the iNH mutant (Fig. 2B, lane 2), and 3.2- and 2.7-kb bands in the aadAΔH mutant (Fig. 2B, lane 1). Hybridization to the common 3.2-kb band is stronger because the probe has more sequence homology with it. Southern-blot analyses with the mutants iUN and iHE using similar probes also established the approximate location of the antibiotic cassette in these strains (data not shown).

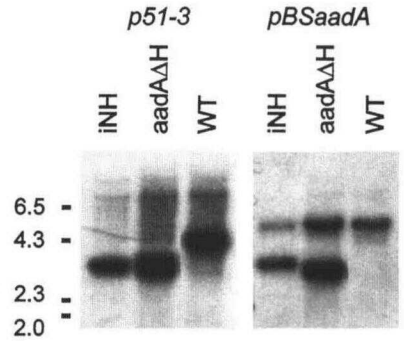

Figure 2. Southern-blot analyses of the *aad*A cassette insertion site. Total DNA from wild-type (WT), mutant iNH, and mutant aadAΔH were digested with *Eco*RI and subjected to Southern-blot analyses with radiolabeled probes derived from plasmids p51–3 and pBSaadA. With the latter, the DNA fragment of approximately 6 kb found in the mutants and wild type is due to hybridization with the endogenous *rbc*L/*atp*A sequences.

To further characterize the insertion site of the cassette in the constructs, PCR was used to amplify the flanking sequences, which were then sequenced. This revealed that the iNH mutant has the *aad*A cassette inserted at a position corresponding to nucleotide 2270 of clone 51–3 (Johnson and Schmidt, 1993). In mutant aadAΔH the cassette replaces positions 2270 to 2651. Thus, 381 bp of the chloroplast genome, including the entire *psb*H coding region, are deleted and replaced by the 1.9-kb *aad*A cassette. PCR was similarly employed to confirm the precise site of insertions in iUN and iHE (data not shown).

Northern-Blot Analyses

Previous work has shown that the accumulation of the dicistronic *psb*B/T transcript and several lower-molecular-weight transcripts possessing the *psb*H coding sequence are coordinately regulated in *C. reinhardtii* (Sieburth et al., 1991; Monod et al., 1992; Johnson and Schmidt, 1993). Thus, the nuclear mutants GE2.10 and 222E lack all of these transcripts as the result of rapid degradation processes that occur in the absence of a nuclear-encoded, highly specific RNA stabilization or preRNA maturation factor. To determine whether there is an interdependence of *psb*B/T and *psb*H mRNA accumulation in mutant aadAΔH and whether anomalous transcripts occur when the gene cluster is interrupted in mutant iNH, northern-blot analyses were performed with RNAs from these strains (Fig. 3). Probes for *psb*A, *psb*C, and *psb*D show that there are no effects of the aadAΔH or iNH mutations on the levels of these chloroplast transcripts. Similar results were obtained with a probe for *psb*N and RNA from mutants iUN and iHE. In contrast, both aadAΔH and nuclear mutant GE2.10 lack detectable amounts of high- and low-molecular-weight species of *psb*H mRNA. GE2.10 also accumulates no *psb*B/T mRNA because this transcript, as well as that of *psb*H, is rapidly degraded after synthesis in this mutant (Sieburth et al., 1991). The data indicate that anti-sense sequences upstream of *psb*H have no effect on the levels of this mRNA and, from mutant iUN, that the strong *rbc*L terminator also does not diminish *psb*H transcripts. It appears most likely that in *C. reinhardtii*, *psb*H has its own promoter and that the absence of both *psb*B/T and *psb*H mRNAs in mutants GE2.10 and 222E result from defective RNA stabilization protein(s) interacting with similar motifs in the individual gene products. Moreover, accumulation of *psb*B/T mRNA is completely independent of that of *psb*H mRNA. Thus, the photosynthetic deficiency of mutant aadAΔH cannot be explained on the basis of an absent mRNA for the CP47 apoprotein.

Thylakoid Proteins

Proteins of the PSII complex are selectively affected in the aadAΔH mutant, as shown in stained gels of thylakoids (Fig. 4A). Conspicuous in stained gels of thylakoid proteins of wild-type cells (and of the mutants iUN, iNH, and iHE) are the apoproteins of CP47 (PSII-B) and CP43 (PSII-C). These proteins of 48 and 43 kD in *C. reinhardtii* are absent in mutant aadAΔH. The extrinsic lumenal proteins OEE24 and OEE16 of the PSII water-splitting complex are also absent from aadAΔH thylakoids because these are released from the thylakoid lumen during purification from mutants lacking PSII reaction centers (Greer et al., 1986). In contrast, there is no effect of the mutation on the levels of proteins of other photosynthetic complexes. The two major proteins of the PSII reaction center, D1 (PSII-A) and D2 (PSII-D), are Lys-deficient and therefore are not readily

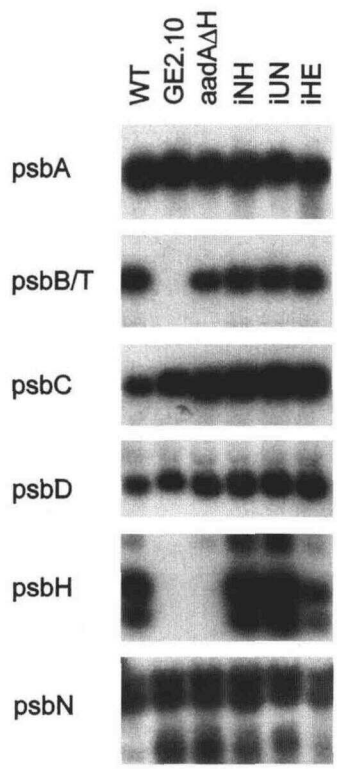

Figure 3. Chloroplast transcripts of PSII genes. Total RNA from wild-type and the indicated mutant cells were subjected to northern-blot analyses with probes corresponding to the indicated chloroplast genes.

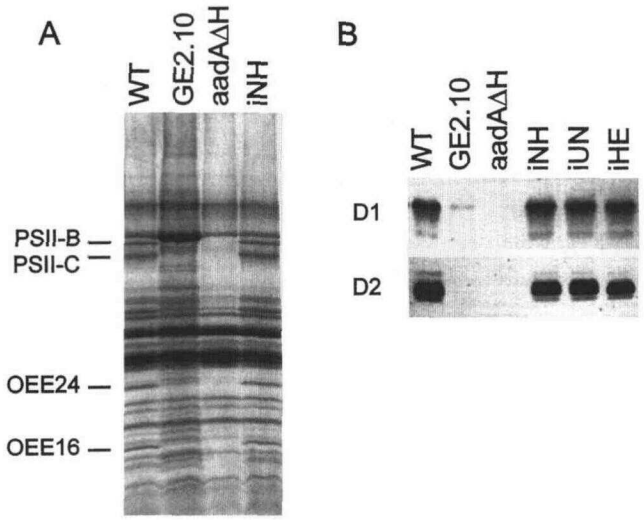

Figure 4. Thylakoid protein analyses. A, Stained gel profile of thylakoid proteins of wild type and mutants GE2.10, aadAΔH, and iNH. The positions of conspicuous PSII proteins are indicated. B, Immunoblots of thylakoid proteins from the same strains, as well as mutants iUN and iHE.

detected by protein staining. However, immunoblot analyses demonstrate that D1 and D2 are depleted in mutant aadAΔH (Fig. 4B), although they are detected in trace amounts when gels are overloaded (data not shown). The PSII-deficient phenotype of mutant aadAΔH is unchanged when cells are grown heterotrophically in the dark (not shown). Therefore, instability of PSII through enhanced sensitivity to photodamage does not account for the characteristics of mutant aadAΔH. The results indicate that PSII-H could be necessary for the assembly and accumulation of other subunits of PSII. Alternatively, PSII-H might participate in the control of translation of mRNAs encoding other PSII proteins and failure to produce these components would result in the absence of the complex.

To evaluate the effect of elimination of *psb*H on the synthesis of other chloroplast-encoded proteins, cells were subjected to pulse-chase labeling in the presence of cycloheximide. As seen in a comparison of results obtained with wild-type and aadAΔH cells in Figure 5, the only ^{35}S-labeled protein in which synthesis is greatly impaired in mutant aadAΔH is approximately 10 kD, the approximate size of PSII-H. D1, D2, PSII-B, and PSII-C are pulse-labeled at levels approaching that of wild-type cells. Therefore, the sequences eliminated in aadAΔH are also dispensible for translation of *psb*B/T mRNA. In chase periods of 2 and 4 h, PSII-H of wild-type cells appears to undergo further modification such that two derivatives of slightly lower electrophoretic mobility arise. We tentatively attribute these electrophoretic mobility shifts to phosphorylation at two sites in newly synthesized PSII-H. Other proteins that undergo electrophoretic mobility shifts correspond to D2 and a polypeptide that may be a component of the Cyt b_6/f complex. D2 appears as a doublet in mutant aadAΔH as well as wild-type cells. This result indicates that phosphorylation of D2 does not involve PSII-H either as an activator for protein kinase(s) or as a substrate for a phosphotransferase.

It is apparent that turnover of the remaining PSII subunits is rather slow when the PSII-H protein is not synthesized. Data obtained from densitometric scans of autoradiographs, such as that shown in Figure 5, indicate that in the chase period PSII-B and PSII-C are not perceptibly affected, whereas D1 and D2 levels are decreased by 20 and 40%, respectively. Equivalent results to those shown in Figure 5 were obtained when chloramphenicol was employed to block further chloroplast protein synthesis, supplementing the effect of excess unlabeled sulfate in the chase period (data not shown). Ultimately, degradation of the major PSII proteins must occur in the absence of PSII-H, because they are not detected in stained gels or immunoblots of aadAΔH thylakoids. Nonetheless, the turnover rate of these proteins is much slower in aadAΔH than in mutants in which the synthesis of other PSII subunits is specifically affected.

Role of PSII-H in PSII Assembly

To evaluate the participation of PSII-H in biogenesis of PSII, cells of aadAΔH and wild type were subjected to 20 min of pulse-labeling with $^{35}SO_4$ in the presence of cycloheximide. Subsequently, thylakoids were solubilized with *n*-dodecyl-β-D-maltoside, and photosynthetic complexes

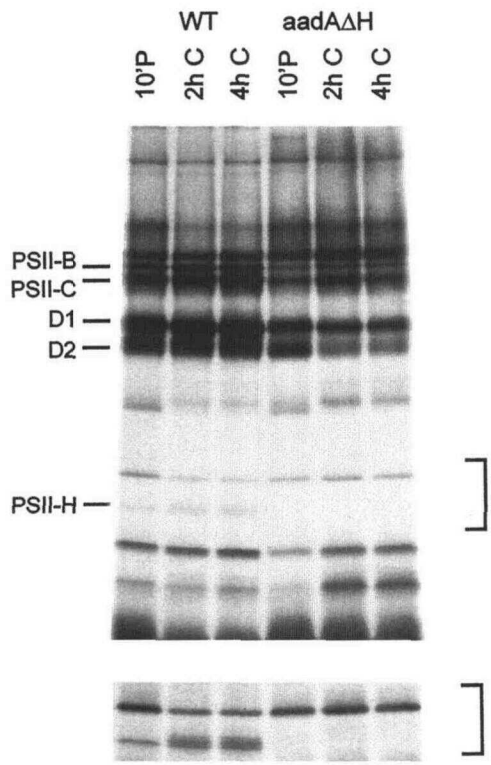

Figure 5. Pulse-chase labeling analyses. Shown is a fluorograph of thylakoid proteins radiolabeled in the presence of cycloheximide during pulse-chase analyses of wild-type and mutant aadAΔH. Samples of 10,000 cpm were loaded in each lane. The positions of the PSII core proteins are indicated. The lower portion of the figure is a longer exposure of the bracketed region of the gel showing the appearance of a doublet of PSII-H proteins in wild-type thylakoids, which is absent in mutant aadAΔH.

were fractionated by Suc gradient centrifugation. As seen in Figure 6, newly synthesized PSII proteins from wild-type thylakoids sediment broadly through the Suc gradient but are recovered most abundantly in fraction 6, where PSII-H is most conspicuous. In contrast, the pulse-labeled PSII core proteins from mutant aadAΔH are mostly in fraction 3; diminishing amounts are also found in fractions 6 and 7 but not in more dense regions of the gradient. A more quantitative depiction of the differences in PSII assembly is presented in Figure 7. The bulk of radioactivity in fraction 6 of wild-type proteins is of PSII origin (Fig. 6), whereas the yield from pulse-labeled thylakoids of mutant aadAΔH not only is quite small, but is also masked by large amounts of α- and β-subunits of co-sedimenting ATP synthase (Fig. 6). Overall, the data indicate that assembly of PSII can proceed in the absence of PSII-H. Without PSII-H, however, either this is a slow process, the complexes are labile under the conditions of ultracentrifugation, or assembly is followed by dissociation in vivo. The last possibility would be consistent with a failure of the PSII core proteins to accumulate in aadAΔH. In any case, it is clear that PSII-H is vital to the structural integrity of PSII.

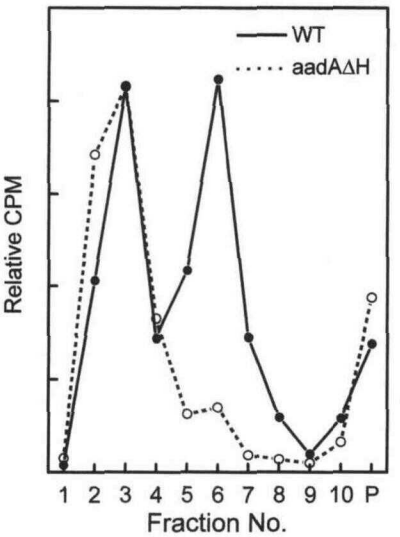

Figure 7. Distribution of radioactivity in Suc gradient fractions of pulse-labeled thylakoids. Aliquots of samples obtained from Suc gradient fractionation of pulse-labeled thylakoid proteins, as described in the legend for Figure 6, were subjected to scintillation counting. The plot was normalized for equivalent cpm from fraction 3 for the mutant and wild-type samples.

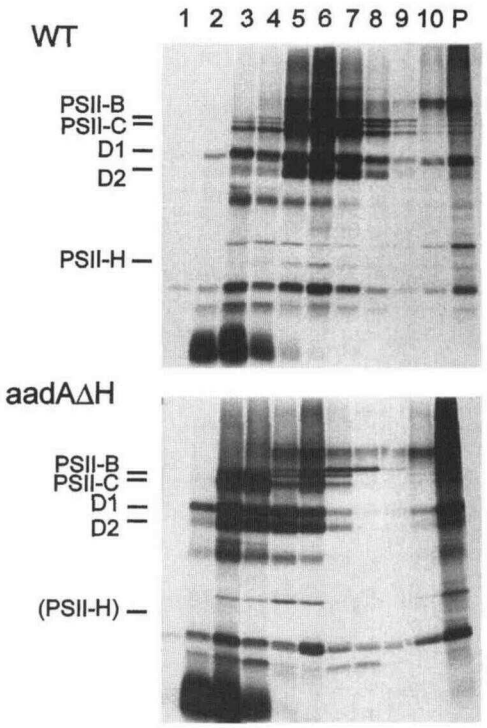

Figure 6. Suc gradient fractionation of pulse-labeled thylakoid proteins. Cells of mutant aadAΔH and wild type were subjected to 20 min of pulse-labeling with $^{35}SO_4$ in the presence of cycloheximide before thylakoids were isolated and subjected to Suc gradient fractionation after solubilization with *n*-dodecyl-β-D-maltoside. Fractions were collected from top to bottom as indicated and, together with pelleted material (P), were subjected to SDS-PAGE and fluorography. The positions of PSII core proteins are indicated except in the gel of proteins from mutant aadAΔH, where (PSII-H) refers to the expected position of this protein if it were synthesized in this strain.

DISCUSSION

Unlike the case of the cyanobacterium *Synechocystis* (Mayes et al., 1993; Komenda and Barber, 1995), PSII-H is necessary for the accumulation of the PSII core proteins in *C. reinhardtii*. These results complement and extend another report of a *Chlamydomonas psb*H deletion mutant in which the absence of PSII activity was assessed by oxygen evolution, electron paramagnetic resonance, and fluorescence spectroscopy (Ruffle et al., 1995). The disparity between the requirement of PSII-H in cyanobacteria and *C. reinhardtii* could be due to the extensive N-terminal sequence differences of the protein of these species, resulting in evolutionary divergence in its physiological role(s). On the other hand, there are now many documented cases for differences in the requirements of subunits for PSII stabilization in cyanobacteria versus *C. reinhardtii* (Vermaas, 1993). Recent examples include PSII-K (Ikeuchi et al., 1991; Zhang et al., 1993; Takahashi et al., 1994) and PSII-I (Ikeuchi et al., 1995; Kunstner et al., 1995), small PSII proteins that affect biogenesis or stability of the complex in a manner considerably different in cyanobacteria than in *C. reinhardtii*, a green alga. PSII-I, another low-molecular-weight polypeptide that is intimately associated with the D1-D2-Cyt b_{559} reaction center (Ikeuchi and Inoue, 1988), is dispensible in cyanobacteria (Ikeuchi et al., 1995) and in *C. reinhardtii* grown under dim light (Kunstner et al., 1995). Thus, it is not possible to define structural/functional properties of the small polypeptides simply on the basis of either their proximal or distal location in the PSII complex.

The function of PSII-H, and the significance of its light-dependent phosphorylation, is a long-standing mystery. Packham (1988) proposed that PSII-H is the PSII counterpart to the H subunit of reaction centers of photosynthetic

purple bacteria even though they exhibit little amino acid sequence similarity. Accordingly, he suggested that PSII-H supports plastoquinone interaction and reduction at the Q_B site and that its phosphorylation would change the conformation of the Q_B pocket to reduce plastoquinone binding. Partial support for this idea was originally provided by Shochat et al. (1982) and Vermaas et al. (1984), who found that PSII phosphorylation affects herbicide affinity and that phosphorylated thylakoids exhibit decreased rates of electron transport from Q_A to Q_B (Packham et al., 1988). Horton and Lee (1984) monitored an increase of in vitro cyclic electron transport around PSII in phosphorylated membranes that correlated with a decrease in the capacity for linear electron flow under light-saturating conditions. To date, it has not been determined if these phenomena stem from the formation of a single PSII phosphoprotein such as PSII-H, or if they arise from concerted phosphorylation processes. Replacement of *psb*H with a gene mutated at phosphorylation site(s) should aid in resolution of this problem. In fact, such a mutant has been constructed with an N-terminal substitution of Ala for Thr: it was reported to exhibit no phosphorylation of PSII-H or other PSII proteins in an in vitro assay with purified thylakoids (Cheater et al., 1995). Because phosphorylation of light-harvesting proteins was also severely affected, in vivo phosphorylation studies are needed to confirm a central role of PSII-H in regulating protein kinase activities.

On the basis of thermoluminescence and fluorescence decay studies of a mutant of the cyanobacterium *Synechocystis*, Mayes et al. (1993) concluded that PSII-H is important for maximizing Q_A to Q_B electron transport on the reducing side of PSII. Subsequently, Komenda et al. (1995) determined that the absence of PSII-H promotes photodamage of the D1 protein but also somehow results in reduced rates of its degradation compared with wild-type cells. Together, these studies indicate that PSII-H could function to regulate electron transport between the quinone binding sites of the reaction center, perhaps by facilitating quinone/quinol exchange at Q_B. If this were the case, a reversible interaction of PSII-H with the D1 protein might occur to effect conformational changes at the Q_B pocket. Under photoinhibitory conditions, PSII-H/D1 interactions could therefore enhance degradation of damaged D1. Conformational effects on D1 as a function of PSII-H interactions could also be related to the instability of PSII complexes in *C. reinhardtii*. On the other hand, we note that the PSII protein that is degraded most rapidly in mutant aadAΔH is D2. Thus, a primary interaction with D2, harboring Q_A, can also be considered.

The mode of phosphorylation of PSII-H and the consequence of this modification in the regulation of PSII is unclear. It has been suggested that the cyanobacterial homolog of this protein is not phosphorylated because of sequence truncation and divergence at the N terminus. In chloroplasts an N-terminal Thr, absent in cyanobacterial PSII-H, undergoes this modification after removal of the initiating formyl-Met residue (Bennett, 1991). However, the report of phosphorylation of cyanobacterial PSII-H (Race and Gounaris, 1993) raises the possibility that there are two phosphorylation sites in PSII-H of plants and green algae. This might occur at the region close to the membrane-spanning helix, because considerable sequence homology is found there, including the relative positions of Ser and Thr and of PSII-H from chloroplasts and cyanobacteria. The occurrence of two closely migrating, low-molecular-weight proteins associated with PSII has been reported previously (de Vitry and Wollman, 1988). In pulse-chase labeling studies (Fig. 5), we observe the generation of two polypeptides with slower electrophoretic mobility than the newly synthesized PSII-H of wild-type cells, neither of which are present in mutant aadAΔH. We propose that PSII-H can undergo two phosphorylation events, one of which is at a site that is evolutionarily conserved. This process would partially account for the high degree of phospholabeling of PSII-H, as well as its relatively slow dephosphorylation in darkness (Silverstein et al., 1993).

PSII-H phosphorylation involves a redox-controlled enzyme that seems to differ from the kinase that modifies polypeptides of light-harvesting complexes when the plastoquinone pool is highly reduced (Allen, 1992). Dedner et al. (1988) reported that phosphorylation of PSII-H is much faster ($t_{1/2}$ of 7 min) than that of other PSII proteins ($t_{1/2}$ of 20 min). In addition to the possibility that a unique kinase acts upon PSII-H, it is conceivable that phosphorylated PSII-H is a source for phosphates to be transferred to proteins of the PSII core complex. This hypothesis would extend that of de Vitry et al. (1989), who, from studies of light-harvesting mutants of *C. reinhardtii*, postulated that phosphorylation of PSII proteins involves a light-harvesting/PSII phosphotransferase. In pulse-chase labeling studies with mutant aadAΔH (Fig. 5), however, we observe a mobility shift of D2, documented to correlate with the phosphorylation of this protein (Wollman and Delepelaire, 1984; Ikeuchi et al., 1987b). This result undermines a role of PSII-H as a participant in a phosphotransferase cascade.

An unusual feature of PSII-H is that it accumulates in etioplasts (Hird et al., 1991) as does Cyt b_{559} (Ohashi et al., 1992). Thus, PSII-H and the Cyt b_{559} subunits are refractory to proteolysis in the absence of other PSII proteins. Moreover, roles for these atypical proteins in nucleating or promoting assembly of the reaction center are feasible. Mutation of the *psb*F gene of the Cyt b_{559} β-subunit of *Synechocystis* species results in an almost complete loss of D1 and D2 (Pakrasi et al., 1990), and we observe impaired formation/stability of PSII complexes in mutant aadAΔH (Fig. 6). In the absence of PSII-H, the bulk of newly synthesized PSII proteins are recovered in Suc gradient fractions corresponding to free chlorophyll, although a small fraction reaches the gradient region where wild-type PSII is most abundant. Unfortunately, it is not possible to assess if PSII assembly is initiated normally in aadAΔH cells and then disassembles either in vivo or in vitro. Perhaps PSII-H is necessary for the formation of PSII dimers (Boekema et al., 1995) and, in turn, dimerization is required for long-term PSII stability. In any case, instability of PSII-H-less PSII in vivo is a certainty, because only trace amounts of the proteins of the complex accumulate (Fig. 4). However, turnover of PSII-B, PSII-C, D1, and D2 is much slower than

has been observed in other PSII-deficient mutants of *C. reinhardtii*. For example, in the absence of either PSII-B or D1 synthesis, D2 degradation is nearly complete within 1 h (Jensen et al., 1986). In contrast, substantial amounts of D2 are detected after pulse-chase labeling periods of 2 and 4 h with mutant aadAΔH (Fig. 5). Whereas turnover of PSII proteins in other mutants occurs in a mostly linear fashion, in mutant aadAΔH this process may be more cataclysmic, with most proteins of partially assembled complexes being degraded only after a lag phase that precedes dissociation. Again, although D2 is the most rapidly degraded PSII protein in mutant aadAΔH, it is still uncertain whether this indicates an intimate relationship between PSII-H and D2.

The transcripts of *psb*B/T and *psb*H are rapidly degraded in *C. reinhardtii* nuclear mutants GE2.10 (Sieburth et al., 1991; Johnson and Schmidt, 1993) and 222E (Monod et al., 1992). These observations could be explained by the absence of factors to promote stability of a precursor to the two mRNAs or the absence of factors that bind to stabilization motifs common to *psb*B/T and *psb*H mRNAs if it is supposed that each is independently transcribed. Deletion of *psb*H has no effect on the transcription of the *psb*B/T dicistronic mRNA or accumulation of this transcript (Fig. 3). Interruption of the *psb*B gene cluster, exemplified in mutant iUN, similarly does not affect the accumulation of *psb*H mRNA even though the *rbc*L transcriptional terminator is known to be highly effective in vivo (Blowers et al., 1993). A similar deduction can be reached from the results of disruption of the *psb*T sequence in *C. reinhardtii* (Monod et al., 1994). Therefore, it is most likely that transcription of *psb*B/T and *psb*H can occur independently of one another in *C. reinhardtii*. This result is consistent with the failure to detect a *psb*B/T-*psb*H pre-mRNA in cells grown at low temperatures (Johnson and Schmidt, 1993). Also, promoter motifs have been identified in upstream sequences at appropriate distances from the 5′ terminus of at least one of the four major *psb*H transcripts of *C. reinhardtii* (Johnson and Schmidt, 1993). Consequently, we favor independent transcription of *psb*B/T and *psb*H in the alga, but cannot eliminate the possibility that co-transcription also can occur. There are numerous examples for alternative promoters in chloroplasts, including those for *pet*D in *C. reinhardtii* (Sturm et al., 1994).

Finally, the pulse-chase labeling studies with mutant aadAΔH demonstrate that PSII-H is not involved in regulating translation of *psb*B/T or any other protein of the PSII core complex. This finding is of importance because a secondary effect of the GE2.10 mutation, entailing strong attenuation of *psb*A translation, was attributed to the absence of PSII-B at a time when it was not known that PSII-H is also not synthesized in the mutant (Jensen et al., 1986). Thus, the model of Jensen et al. (1986), invoking feedback interactions dependent upon the presence of unassembled PSII-B for *psb*A translation, remains valid.

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