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The 9-kDa phosphoprotein of photosystem II. Generation and characterisation of *Chlamydomonas* mutants lacking PSII-H and a site-directed mutant lacking the phosphorylation site

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Abstract

The chloroplast gene *psb*H encodes a 9–10 kDa thylakoid membrane protein (PSII-H) that is associated with photosystem II and is subject to light-dependent phosphorylation at a threonine residue located on the stromal side of the membrane. The function of PSII-H is not known, neither is it clear what regulatory role phosphorylation may play in the control of PSII activity. Using particle gun-mediated transformation, we have created chloroplast transformants of *Chlamydomonas reinhardtii* in which the synthesis of PSII-H is prevented by the disruption of *psb*H, or in which the phosphorylatable threonine is replaced by alanine through site-directed mutagenesis of the gene. The mutants lacking PSII-H have a photosystem II-deficient phenotype, with no detectable functioning PSII complex present in whole cells or isolated thylakoid membranes. In contrast, the alanine mutant (T3A) grows photoautotrophically, and PSII activity is comparable to wild-type cells as determined by various biochemical and biophysical assays.

Keywords: Phosphoprotein; Photosystem II; *psb*H; PSII-H; Mutagenesis; *(Chlamydomonas)*

1. Introduction

Photosystem II (PSII) is a pigment–protein complex located in the thylakoid membranes of cyanobacteria, algae and higher plants that contains more than 20 polypeptide subunits [1]. It catalyses the light-induced vectorial electron transfer from water to plastoquinone that leads to oxygen evolution and production of an electrochemical gradient across the membrane. The core of the complex consists of two co-factor binding proteins termed D1 and D2, together with the heterodimeric cytochrome b_{559} and

Abbreviations: PSII, Photosystem II; Chl, Chlorophyll; EPR, Electron paramagnetic resonance; PQ, Plastoquinone; DCMU, 3-(3,4-Dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transfer from Q_A to Q_B in PS II; EDTA, Ethylenediamine tetraacetic acid, a chelator of bivalent metal ions; LHCII, Light harvesting chl a/b protein complex of photosystem II; PAR, Photosynthetically active radiation; TAP, Tris-acetate phosphate growth medium

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several small polypeptides. Two chlorophyll binding polypeptides, CP47 and CP43, form an inner antennae complex. A number of other polypeptides of unknown function are associated with PSII including the *psb*H gene product, PSII-H [2].

A unique feature of thylakoid membranes is reversible thylakoid protein phosphorylation under light-dependent redox control [3]. Protein phosphorylation is a ubiquitous mechanism for signal transduction and control of protein function. Thylakoid protein phosphorylation was first reported 20 years ago [4]. The major phosphoprotein was identified as the light harvesting chl a/b protein complex (LHCII) [5]. The thylakoid protein kinase is membrane-bound, activated in the light or in the presence of strong reductants and requires Mg^{2+} as a co-factor [6]. The protein phosphorylation is reversed in the dark by a membrane-bound phosphatase, whose activity is independent of light and redox potential, and is inhibited by fluoride [7]. In addition to LHCII, several PSII-associated proteins are seen to undergo phosphorylation [8]. The most prominent of these is the 9–10 kDa component, PSII-H, which is phosphorylated at a threonine at position 2 in the mature protein [9]. This phosphorylation site is followed by a region of basic residues, and is thought to be exposed to the stromal side of the membranes [7,10]. PSII-H is the second most heavily phosphorylated thylakoid protein after LHCII, and its light-dependent phosphorylation appears to be under similar redox control, although dephosphorylation of PSII-H occurs at a very much slower rate [11].

The function of PSII-H and the regulatory significance of its phosphorylation are uncertain. Thylakoid protein phosphorylation has been demonstrated to affect electron transfer through PSII as shown by an ATP-induced inhibition in oxygen evolution capacity [12,13]. Packham [14] attributed this to PSII phosphorylation, and this was supported by Harrison and Allen [15]. It was suggested that these effects could be due to PSII-H, and that an increase in negative charge due to protein phosphorylation could lead to a stabilization of Q_A in the light and slow electron transfer from Q_A to Q_B. Further studies by Hodges et al. [16] showed that the initial Q_A to Q_B electron transfer is not affected by phosphorylation but that the slow re-oxidation phase of Q_A semiquinone was affected. It was concluded that protein phosphorylation might alter the equilibrium between Q_B and the PQ pool. A direct phosphorylation effect on Q_B binding is not thought to occur as the ability of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to inhibit electron transfer is not altered by phosphorylation [13,15].

The phosphorylation induced decrease in electron transfer through PSII could be a response to photoinhibition [17,18]. Kuhn et al. [19] observed a selective loss of PSII-H upon high light treatment of spinach PSII, and suggested that PSII-H is involved in an early event in photoinhibition. Sundby et al. [20] observed that 20 mM bicarbonate provides protection from photoinhibition at the same time as selectively decreasing phosphorylation of PSII-H in isolated spinach thylakoids. Sundby et al. [20] suggested that the N-terminal cluster of basic amino acids might provide a binding site for the bicarbonate anion, which would account for competition between bicarbonate and phosphorylation.

The green unicellular alga *Chlamydomonas reinhardtii* represents a useful eukaryotic model for studies of photosynthesis in higher plants. Photosynthetic mutants of *C. reinhardtii* are viable, since the alga is capable of heterotrophic growth using acetate as a carbon source, and are amenable to biochemical and biophysical analysis. Furthermore, the genetic engineering of the chloroplast-encoded components of the photosynthetic apparatus is a routine procedure, employing microparticle bombardment to deliver cloned DNA into the chloroplast genome [21]. This approach has been successfully employed to disrupt or modify a large number of photosynthetic genes in *C. reinhardtii* [22]. The availability of the *C. reinhardtii* *psb*H gene sequence [23,24], and the phosphorylation of the N-terminal protein sequence [25,26] provides an opportunity to investigate the role of the protein in the functioning and regulation of PSII in eukaryotes. In cyanobacteria, Mayes et al. [27] deleted the *psb*H gene in *Synechocystis* 6803 and obtained a mutant capable of photoautotrophic growth at low light intensity, but not at higher light intensities. A role for PSII-H in photoinhibition was suggested after further characterisation of this mutant [28]. Extrapolation from studies of cyanobacterial *psb*H mutants to eukaryotes are complicated since: (i) the N-terminal phosphorylation site is absent in the 6.5 kDa cyanobacterial protein, although Race and Gounaris

[29] suggest that *Synechocystis* PSII-H may still undergo phosphorylation at an alternative site; (ii) cyanobacteria lack LHCII, and therefore the mechanisms and regulatory significance of the light-dependent phosphorylation of LHCII and PSII-H cannot be understood using a bacterial model; (iii) The assembly and/or stability requirements of the PSII complexes appear to differ markedly in *C. reinhardtii* and cyanobacteria. For example, deletion of the *psb*K or *psb*N genes in cyanobacteria still results in the formation of a functional PSII complex [30]. This is not the case in *C. reinhardtii*, where deletion of the gene results in complete loss of photoautotrophic growth ([31], our unpublished data).

We report here the generation and analysis of PSII-H mutants of *C. reinhardtii*. Mutants lacking the protein are unable to assemble any functional PSII, even in the dark, and consequently are incapable of photoautotrophic growth. However, replacement of the phosphorylatable threonine with alanine results in mutants that are essentially indistinguishable from wild-type cells, as judged by several biochemical and biophysical assays. Preliminary reports of this work have been presented as conference proceedings [32,33], and while this manuscript was in preparation, Summer et al. [34] reported similar findings on the insertional inactivation of *psb*H in *C. reinhardtii*.

2. Materials and methods

2.1. Strains and culture conditions

The wild-type *C. reinhardtii* strain 2137 mt + [35] was obtained for the *Chlamydomonas* Culture Center, Duke University, where it is catalogued as CC-1021. Strain B4, a nuclear PSI-deficient mutant, was a gift from L. Mets (University of Chicago). Cells were grown on Tris–acetate phosphate (TAP) medium or high salt minimal (HSM) medium [36], supplemented with spectinomycin where required [37]. For growth studies, cells were cultured at 25°C under an illumination of 50 μE m^{-2} s^{-1} photosynthetically active radiation (PAR). For molecular biological and biochemical analysis, cells were grown in TAP liquid medium at 25°C in dim light (6–8 μE m^{-2} s^{-1} PAR) with aeration. Chlorophyll assays were performed on whole cells and thylakoid membranes according to the methods of Arnon [38] and Porra et al. [39].

For thermoluminescence, fluorescence induction and flash-induced oxygen yield experiments, cells were harvested by gentle filtration with Whatman microfibre disks (GF/C 25 mm diameter) to give 50 μg chl/disk. For measurements, 400 μl of fresh TAP medium was applied to the cells on the disk surface.

2.2. DNA cloning and site-directed mutagenesis

Recombinant DNA techniques were according to standard protocols [40] using *E. coli* XL1-blue (Stratagene) as the cloning host. Plasmid P-72A containing the 4.4 kb *Eco*RI fragment (Eco19) from the *C. reinhardtii* chloroplast genome inserted into pUC8 was obtained from the *Chlamydomonas* Genetics Center. The *psb*H gene on P-72A was disrupted at the twentieth codon by inserting the *aadA* cassette (in both orientations by blunt end cloning) as a 2 kb *Eco*RV-*Sma*I fragment from plasmid pUC-*atpX*-AAD [37] into the unique *Bst*XI site within the *psb*H coding region. Similarly, the cassette was inserted into the unique *Mlu*I site downstream of *psb*H in the opposite orientation to the gene. This plasmid was then used to introduce a site-directed change at the third codon in *psb*H. A two-step PCR procedure [41], in which the region between the *Pac*I and *Not*I sites (Fig. 1) was replaced with the PCR product, was used to change the sequence ATGGCAACAGGA to ATGGCT*GCA*gGA, such that the threonine codon is replaced with a codon for alanine (italicised) and a *Pst*I site is introduced into the DNA (underlined). All plasmid constructs were confirmed by restriction digest and DNA sequencing.

2.3. Generation of chloroplast transformants

Chloroplast transformation of strain CC-1021 was carried out exactly as described by Hallahan et al. [42]. Transformants were taken through at least three rounds of single colony isolations on TAP medium +100 μg/ml spectinomycin to obtain homoplasmic lines, in which all copies of the chloroplast genome contain the *aad*A cassette. This was confirmed by Southern blot analysis of miniprep DNA [36] using

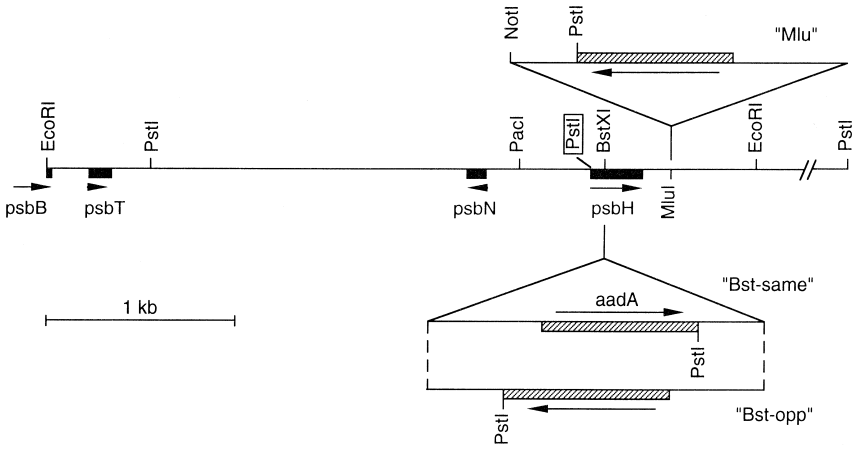

Fig. 1. Mutagenesis of *psb*H. The position and direction of transcription of *psb*H and neighbouring PSII genes on the *C. reinhardtii* chloroplast genome are indicated. The *aad*A cassette was inserted into the *Bst*XI site in both directions to create the disruption mutants, and into the downstream *Mlu*I site to generate the control transformant (MluI) and the T3A mutant. In the latter, a novel *Pst*I site (boxed) is introduced into *psb*H.

radiolabelled Eco19 and *aad*A DNA as probes. Introduction of the *Pst*I site in the site-directed mutant was confirmed by Southern analysis using a 0.7 kb *Dra*I fragment spanning *psb*H. Transformants were maintained on TAP medium in dim light (<5 μE m^{-2} s^{-1} PAR).

2.4. Preparation of C. reinhardtii thylakoid membranes

The method used to obtain photosynthetic membranes from *C. reinhardtii* was a combined modified method of Diner and Wollman [43] and Shim et al. [44]. Throughout the extraction procedure, every step was taken to ensure that the cells and membranes were exposed to minimum light, and were kept at 4°C. The cultures were harvested, pelleted by centrifugation at $5000 \times g$ for 5 min at 4°C, washed once in HSM buffer (20 mM Hepes, 0.35 M sucrose, 2 mM $MgCl_2$, pH 7.5) and then resuspended to a chlorophyll concentration of 1 mg ml^{-1} and passed once through a French press at 4000 lb in^{-2}. The broken cells were then spun at $50\,000 \times g$ for 30 min at 4°C. The pellet was resuspended in 2.2 M sucrose buffer (2.2 M sucrose, 5 mM Hepes, 10 mM EDTA, pH 7.5) to give a final sucrose concentration of 1.8 M sucrose, overlaid with 0.5 M sucrose buffer (5 mM Hepes, 0.5 M sucrose, pH 7.5) and centrifuged for 2 h at $100\,000 \times g$. The photosynthetic membranes that float at the interface of the two sucrose buffers were collected and washed once in thylakoid buffer A (0.4 M sucrose, 20 mM Hepes, 5 mM $MgCl_2$, pH 7.5) and finally resuspended in buffer A. The membranes were stored frozen in liquid nitrogen.

2.5. Oxygen evolution analysis

Steady-state oxygen evolution studies were performed on cells and membranes using a Clark type electrode. Fifty micrograms total chlorophyll in 3 ml of resuspending medium, (20 mM MES, 15 mM NaCl, 5 mM $MgCl_2$ pH 6.3) was used. The electron acceptors used were 2,6-dimethylbenzoquinone (final concentration 1 mM) and potassium ferricyanide (final concentration 1 mM). An average value from duplicate measurements on several sets of cultures of the same cell type is shown.

2.6. EPR analysis of C. reinhardtii cells and membranes

0.3 ml of packed cells or concentrated membranes were placed in a 3-mm diameter quartz EPR tube, dark adapted for 30 min and frozen in liquid nitrogen in the dark. EPR analysis was performed using a Jeol REIX spectrometer with Oxford Instrument liquid helium cryostat.

2.7. Flash-induced oxygen yield

The flash dependence of oxygen evolution was measured with an unmodulated bare-platinum electrode as described in Ref. [45]. Oxygen evolution was induced by a series of short (3 ms) flashes provided by a General Radio Stroboslave 1539-A Xenon flash. Signals were detected by a home-built amplifier and an IBM PC based data acquisition system. Oxygen evolution was induced by 20 flashes, given with 1 Hz frequency. Where indicated, 1 mM 2,5-dichlorobenzoquinone was added at the beginning of the dark adaptation period.

2.8. Thermoluminescence

Thermoluminescence was measured with a home-built apparatus as described previously [27]. After 3-min dark adaptation at 20°C, the samples were excited by a single saturating flash, provided by the 1539-A Xenon flash lamp, at −10°C. This was followed by a fast cooling to −40°C, from where the slow heating with a rate of 20°C min^{-1} was initiated, and thermoluminescence was detected. Where indicated, 10 μM DCMU was added in the dark prior to the dark adaptation.

2.9. Fluorescence

Fluorescence induction was recorded up to 3 s in the absence and presence of 10 μM DCMU. For fluorescence relaxation measurements, cells were illuminated with a single saturating flash. The relaxation kinetics of fluorescence yield after the flash was followed from 150 μs to 30 ms with a 34 ms time resolution. The signal-to-noise ratio was improved by accumulating 20 traces measured at 20 s intervals on the same sample. Cells were also analyzed using the Plant Efficiency Analyzer (Hansatech Instruments). A light intensity of 50% maximum was used with an illumination time of 15 s.

3. Results

3.1. Construction of PSII-H mutants of C. reinhardtii

Mutants unable to synthesize PSII-H were created by chloroplast transformation in which the endogenous *psb*H gene sequence was replaced with *psb*H disrupted by insertion of the spectinomycin resistance marker, *aad*A [37]. Two classes of transformant (termed 'Bst-same' and 'Bst-opp') were generated by inserting the *aad*A into the unique *Bst*XI site within the *psb*H coding region, in the same or opposite direction to *psb*H (Fig. 1). Transformants were taken through three rounds of clonal selection on spectinomycin to generate homoplasmic lines, in which all copies of the chloroplast genome contain the disrupted *psb*H. This was confirmed by Southern blotting using a probe for *psb*H; the introduction of the *aad*A cassette converting the 13.3 kb *Pst*I fragment of the wild-type genome to two fragments of 11.4 and 3.9 kb (Bst-same) or 12.4 and 2.9 kb (Bst-opp). An example of each class is shown in Fig. 2. Mutants lacking the phosphorylatable threonine at residue two

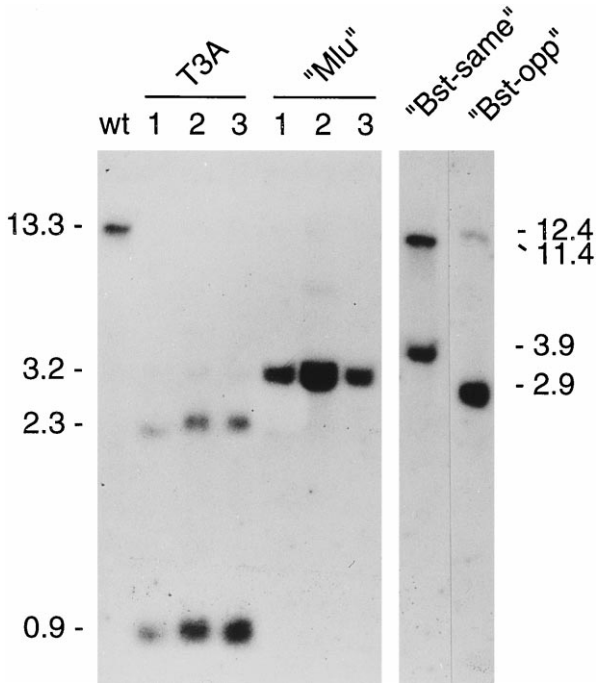

Fig. 2. Southern analysis of transformants. Total DNA was prepared from wild-type (wt) cells, three T3A transformants, three control transformants (Mlu) and a representative of each class of disruption mutant (Bst-same and Bst-opp) as depicted in Fig. 1. *Pst*I-digested DNA was hybridized to a radiolabelled DNA probe specific for *psb*H. In each case, bands of the predicted sizes are obtained (see text). The presence of an additional *Pst*I site in the T3A mutants relative to the Mlu control confirms the threonine to alanine codon change in the mutants. Sizes of bands are given in kilobase pairs.

in the protein sequence [25] were created by placing the *aad*A marker immediately downstream of *psb*H at an *Mlu*I site, and changing the threonine codon (ACA) to that of alanine (GCA) by site-directed mutagenesis. This change also generates a novel *Pst*I site within *psb*H. Again, the introduction of these changes was confirmed by Southern blotting of *Pst*I-digested DNA from the transformants (Fig. 2). In the control transformants ('Mlu') in which *aad*A is inserted downstream of *psb*H but the gene is not altered, the 13.3 kb band is replaced by a band of 3.2 kb. In the site-directed mutants (T3A), this fragment is further restricted by the introduced *Pst*I site to give bands of 2.3 and 0.9 kb. The presence of the *Pst*I site confirms that the *psb*H gene in these transformants contain the threonine-to-alanine codon change.

3.2. Analysis of the PSII-H mutants

Three representatives of each transformant class were chosen for analysis. The phenotype of the transformants was examined by comparing growth in the light on solid medium containing acetate as a reduced carbon source (TAP medium), and on a minimal medium lacking acetate (HSM medium). As shown in Fig. 3, all the transformant lines grew well on TAP and on TAP + spectinomycin, whereas wild-type cells and a photosynthetic mutant strain, B4 were killed in the presence of the drug. In the absence of acetate, B4 failed to grow as did the *psb*H-disruption mutants. However, the T3A mutants and the control transformants grew as well as wild-type, the doubling times being almost equal and showing no photoinhibition up to 250 μE m^{-2} s^{-1} PAR (the maximum attainable in our growth facilities). The same results were obtained for growth in liquid culture; namely, the disruption mutants grew in TAP but not in HSM, whereas the T3A mutants and the control transformants grew as well as wild-type in both media under light intensities of 8 μE m^{-2} s^{-1} and 50 μE m^{-2} s^{-1}. It therefore appears that PSII-H is required for photoautotrophic growth in *Chlamydomonas*. However, the replacement of the phosphorylatable threonine residue with alanine does not prevent photosynthetic function under the light conditions tested. The growth of the *psb*H disruption mutant in *Synechocystis* [27] was inhibited under the higher light conditions.

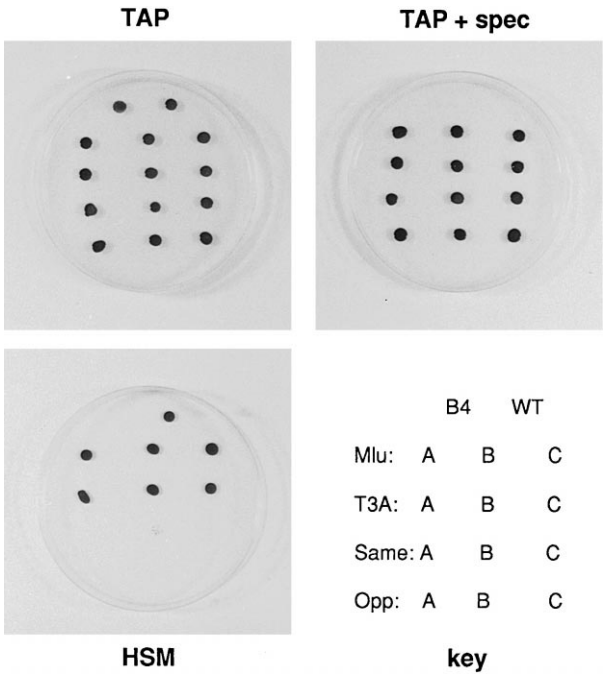

Fig. 3. Growth of transformants. Cultures of wild-type cells, B4 (a photosynthesis-deficient mutant) and three representatives of each transformant class were spotted onto solid medium containing acetate (TAP), medium containing TAP and spectinomycin (TAP + spec) and medium lacking acetate (HSM). Plates were incubated at a light intensity of 45 μE m^{-2} s^{-1} for one week. 'Same' and 'opp' denote the two classes of null *psb*H mutant as described in the text.

3.3. Fluorescence analysis

Variable fluorescence measurements can be used to probe the activity of PSII. The *psb*H mutants were first analyzed using a Plant Efficiency Analyzer (PEA). Analysis of wild-type cells using this method shows an increase (F_V) in fluorescence from F_0 to F_M, then a decline to the steady-state terminal fluorescence. Mutants lacking in a functional PSII show no variable fluorescence. The fluorescence characteristics of MluI and T3A were comparable to that of wild-type cells. Both *psb*H disruption mutants had curves which resemble that of mutants lacking PSII.

The kinetics of fluorescence induction and relaxation were also measured following saturated flashes.

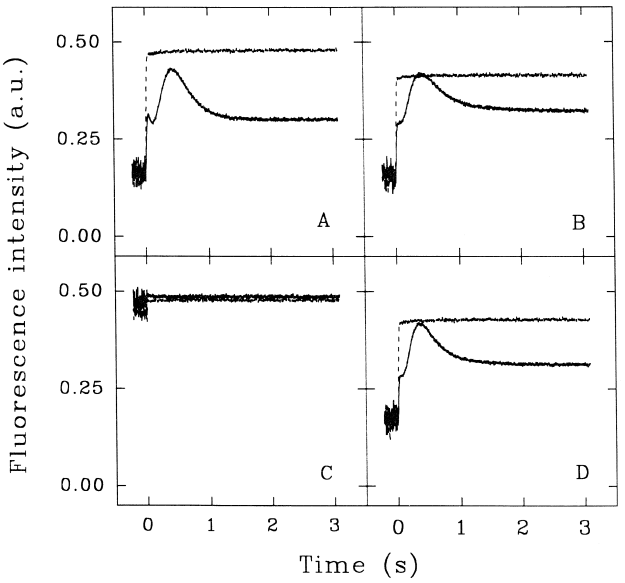

Fig. 4. Fluorescence induction curves measured in the absence (solid line) and presence (dashed line) of DCMU. (A) wild-type (B) MluI (C) *psb*H disruption mutant (D) T3A mutant. See Section 2 for experimental details.

Fluorescence induction and decay curves are shown in Figs. 4 and 5. This is performed with and without the presence of DCMU, a herbicide inhibitor at the Qb site. These measurements provide information on how the electron is shared between the Qa and Qb sites.

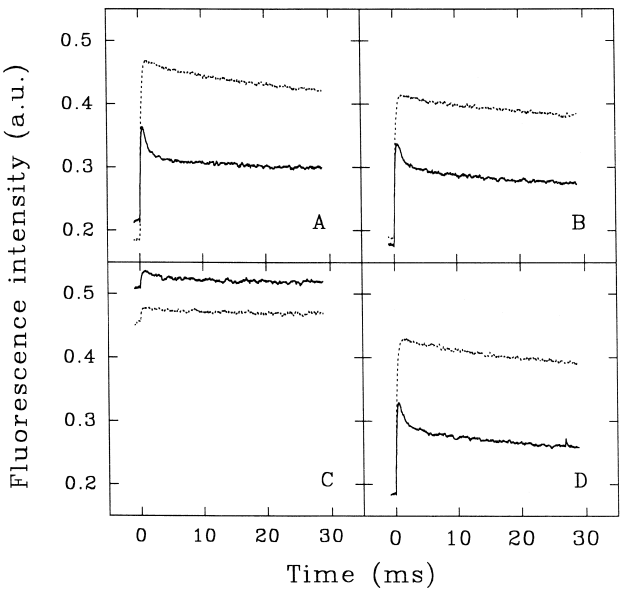

Fig. 5. Fluorescence relaxation curves measured in the absence (solid line) and presence (dashed line) of DCMU. (A) wild-type (B) MluI (C) *psb*H disruption mutant (D) T3A mutant. See Section 2 for experimental details.

The fluorescence induction curve (Fig. 4) shows all the characteristic phases of intact cells. F_V/F_M is about 0.61 without DCMU, and 0.66 with DCMU. Wild-type, MluI and T3A all gave similar results. The *psb*H disruption mutant shows no detectable PSII activity.

For fluorescence relaxation (Fig. 5), more than one phase is observed, an unresolved fast initial phase of Qa- re-oxidation by Qb or Qb-, which is removed by DCMU and a slower phase dependent on occupancy of the Qb site, and/or the QaQb equilibrium. Again wild-type, MluI and T3A all gave similar results, but the *psb*H disruption mutant showed no PSII activity.

It can therefore be inferred that insertion of the *aad*A cassette downstream of *psb*H does not affect the functional integrity of PSII as measured by the F_V/F_M ratios, but insertion into the genome results in apparent loss of PSII activity.

3.4. Thermoluminescence

Thermoluminescence is a useful tool for studying charge stabilization and recombination in PSII. Recombination of positive charges stored in the water-oxidizing complex (S-states) with reduced electron acceptors Q_A and Q_B result in characteristic thermoluminescence emission. The intensity reflects the number of charges and the position of peak temperature is indicative of energy stabilization of charge pair, i.e., increased peak temperature equals increased stability [46]. Samples given one saturating flash after short dark treatment show the B-band seen at 30–40°C from S_2QB^- recombination. In the presence of the herbicide inhibitor DCMU, which blocks the Q_B site the Q band is observed at 10–15°C from $S_2Q_A^-$ recombination [47,48].

Fig. 6 shows the thermoluminescence obtained from wild-type and mutant cells. In wild-type, the B band is at 30–33°C, while the Q band is at 14–16°C. The shoulder near 15°C in the absence of DCMU reflects the presence of Q_A^-. The MluI and T3A cells are similar to wild-type, while the decreased thermoluminescence in the disruption mutant indicate $\ll$ 10%PSII activity.

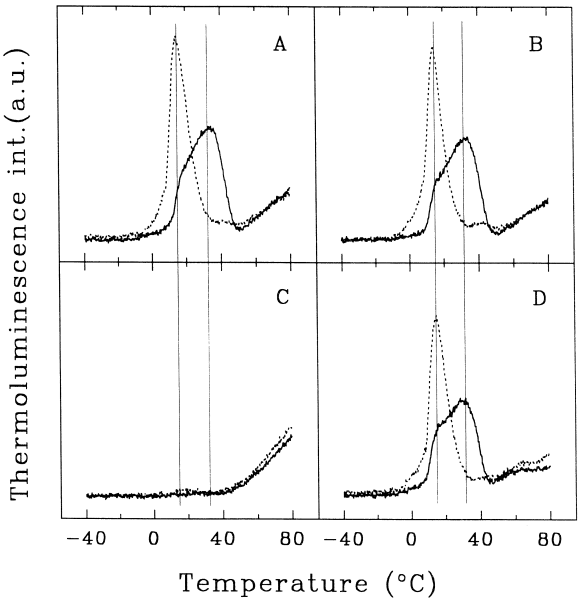

Fig. 6. Single flash thermoluminescence curves measured in the absence (solid line) and presence of DCMU (dashed line) Excitation: 1 flash at −10°C. (A) wild-type (B) MluI (C) *psb*H disruption mutant (D) T3A mutant. See Section 2 for experimental details.

3.5. Oxygen evolution of the psbH mutants

Oxygen evolution was measured in wild-type, MluI, *psb*H disruption mutants and the T3A mutant cells (Table 1). For *psb*H disruption mutants, disrupted in either orientation, no oxygen evolution was recorded indicative of cells lacking PSII. Oxygen evolution was also measured in wild-type, MluI and T3A mutant photosynthetic membranes. The T3A membranes also show a slight but consistent decrease in oxygen evolution compared to wild-type or MluI. Fig. 7 shows the pattern of oxygen yield per flash of cells in the absence of exogenous electron acceptor. Similar patterns with the characteristic maximum on the 3rd flash are observed for all samples, except the *psb*H disruption mutant (not shown), where oxygen evolution is absent.

Table 1
Oxygen evolution characteristics of wild type and mutant cells

Cell type	μmol O_2 /mg chlorophyll per h
Wild type	150
*psb*H deleted	0
MluI	142
T3A	98

Experimental details are given in Section 2.

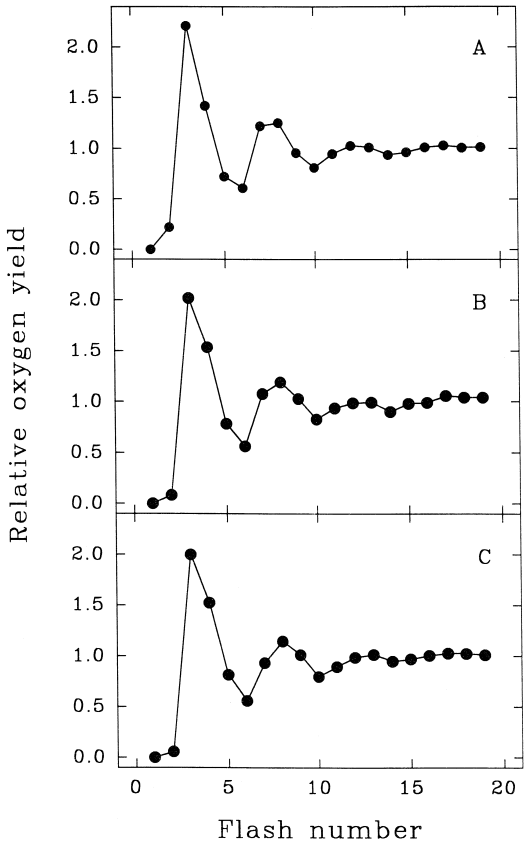

Fig. 7. Flash-induced oxygen yield as a function of flash number. Oxygen evolution was measured with 1 Hz flash frequency. The data are normalised to the average of the 12th to 19th oxygen yield (steady state). (A) wild-type (B) MluI (C) T3A mutant. See Section 2 for experimental details.

3.6. EPR analysis of the psbH mutants

EPR spectroscopy was performed on cells from wild-type *C. reinhardtii* cells in addition to all the *psb*H mutants. In all cases cells were dark adapted for 30 min prior to freezing in liquid nitrogen. In EPR traces from the *psb*H disruption, mutant cells (not shown but see Refs. [32,33]), the Y_D + signal is absent, indicative of a lack of PSII present in the sample. Upon illumination at 12 K, the P700 + signal was observed, indicating that the *psb*H disruption mutants contain functional PSI, and that it is

present in similar amounts to wild-type. EPR traces obtained from whole cells of the mutants T3A and MluI showed identical traces to that of wild type, which confirms the presence of functional PSII.

4. Discussion

The biophysical and biochemical experiments reveal that insertion of the *aad*A cassette into the *psb*H gene in either orientation leads to loss of photoautotrophic growth in *C. reinhardtii* cells. Fluorescence, thermoluminescence, oxygen evolution and EPR analysis all reveal that the *psb*H disruption mutants do not contain functional PSII. EPR analysis reveals that the *psb*H disruption mutants do contain functional PSI, and that it is present in amounts comparable to wild type. The findings presented here, together with the recent work of Summer et al. [34], demonstrate an absolute requirement for PSII-H in the assembly and/or stability of PSII in *Chlamydomonas*. This is in contrast to the situation in *Synechocystis*, in which PSII is able to accumulate in the absence of PSII-H [27]. This difference in the requirement for small subunits for the accumulation of the photosynthetic complexes in these eukaryotic and prokaryotic model systems has been observed for other components of PSII and PSI [31], and may reflect fundamental differences in the mechanisms of complex biogenesis in the chloroplast and the cyanobacterial cell. Based on an analysis of the unassembled PSII component in a mutant lacking PSII-H, Summer et al. [34] suggest that PSII-H facilitates the stable formation of the complex.

Our creation of the site-directed mutant T3A allows for the first time an analysis of the role of PSII-H phosphorylation, as distinct from the function of the protein itself. Interestingly, replacement of the threonine residue with an non-phosphorylatable alanine has no significant effect on photoautotrophic growth and on the functioning of PSII. The possibility that a second threonine residue allows phosphorylation by proxy in the T3A mutant cannot be excluded. Indeed, it has been suggested that the *Chlamydomonas* protein is phosphorylated at several sites [26,34], although a second major site of phosphorylation was not detected in the analysis of the first 42 residues of the wild-type *Chlamydomonas* protein [25]. A preliminary study of the phosphorylation pattern in this mutant [32,33] indicated a general decrease in phosphorylation of PSII polypeptides. The only consistent change in the T3A mutant, however, is the decrease in the rate of oxygen evolution, which indicates that a structural change on the phosphorylation of PSII-H may be transmitted to the reaction center to modify the rate of PSII turnover. Further detailed characterisation of this mutant may reveal the role of *psb*H phosphorylation.

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