Photosystem I Is Indispensable for Photoautotrophic Growth, \( \text{CO}_2 \) Fixation, and \( \text{H}_2 \) Photoproduction in \textit{Chlamydomonas reinhardtii}\*  

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Certain \textit{Chlamydomonas reinhardtii} mutants deficient in photosystem I due to defects in psaA mRNA maturation have been reported to be capable of \( \text{CO}_2 \) fixation, \( \text{H}_2 \) photoevolution, and photoautotrophic growth (Greenbaum, E., Lee, J. W., Tevault, C. V., Blankinship, S. L., and Mets, L. J. (1995) \textit{Nature} 376, 438–441 and Lee, J. W., Tevault, C. V., Owens, T. G.; Greenbaum, E. (1996) \textit{Science} 273, 364–367). We have generated deletions of photosystem I core subunits in both wild type and these mutant strains and have analyzed their abilities to grow photoautotrophically, to fix \( \text{CO}_2 \), and to photoevolve \( \text{O}_2 \) or \( \text{H}_2 \) (using mass spectrometry) as well as their photosystem I content (using immunological and spectroscopic analyses). We find no instance of a strain that can perform photosynthesis in the absence of photosystem I. The F8 strain harbored a small amount of photosystem I, and it could fix \( \text{CO}_2 \) and grow slowly, but it lost these abilities after deletion of \textit{psaA} or \textit{psaC}; these activities could be restored to the F8-psaAΔ mutant by reintroduction of \textit{psaA}. We observed limited \( \text{O}_2 \) photoevolution in mutants lacking photosystem I; use of \( ^{18}\text{O}_2 \) indicated that this \( \text{O}_2 \) evolution is coupled to \( \text{O}_2 \) uptake (i.e. respiration) rather than \( \text{CO}_2 \) fixation or \( \text{H}_2 \) evolution. We conclude that the reported instances of \( \text{CO}_2 \) fixation, \( \text{H}_2 \) photoevolution, and photoautotrophic growth of photosystem I-deficient mutants result from the presence of unrecognized photosystem I.  

The proposal that two different light reactions are involved in oxygenic photosynthesis arose from studies of an “enhancement effect” that was observed when two beams of different wavelengths were used to illuminate algae (3, 4). The discovery of cytochromes \( b \) and \( f \) led to the concept that they served as intermediaries between the two photosystems, which operated in series to achieve linear electron transport from \( \text{H}_2\text{O} \) to NADP\(^+\) (5). The essence of this “Z-scheme” model is that photosystem II (PS II)\(^1\) accomplishes the oxidation of water and the reduction of plastoquinone and cytochromes but is unable to reduce ferredoxin or NADP\(^+\); photosystem I (PS I) is required for the reduction of NADP\(^+\) and the oxidation of cytochrome \( f \) through the mediation of the soluble protein plastocyanin. Although other explanations have been proffered for the enhancement effect and photosystem cooperativity (6), the Z-scheme is strongly supported by experimental data (7) and is currently considered as the “central dogma” of oxygenic photosynthesis (8, 9).  

However, photosynthetic \( \text{CO}_2 \) fixation and photoautotrophic growth were recently reported (1, 2) in two PS I-deficient mutants, B4 and F8, both of which are nuclear mutants deficient in the \textit{trans}-splicing of the \textit{psaA} mRNA (1, 10, 11). Using a gas flow apparatus, Greenbaum \textit{et al.} (1) were able to measure an evolution of \( \text{O}_2 \) coupled to \( \text{CO}_2 \) fixation or to \( \text{H}_2 \) evolution. The amount of \( \text{CO}_2 \) fixed by these mutant strains was roughly equivalent to that measured in wild-type (WT) cells. Although \( \text{CO}_2 \) fixation in the PS I-deficient strains initially appeared to be limited to anaerobic conditions (1), photoautotrophic growth was later reported in the presence of \( \text{O}_2 \) (2). Several control experiments were carried out by the authors to eliminate the possibility of trace amounts of PS I. Irradiation with far red light (\( \lambda > 700 \text{ nm} \)), which excites PS I but not PS II, produced a small amount of \( \text{H}_2 \) evolution in WT cells but not in the B4 mutant (1). Photobleaching experiments also failed to detect \( P_{700} \) in thylakoid membranes from these mutants (2).  

The core of PS I is made up of the two largest subunits, PsaA and PsaB. They bind all of the cofactors involved in intra-PS I electron transport with the exception of the terminal iron-sulfur clusters, which are bound by the extrinsic PsaC subunit. Methods to delete the chloroplast genes \textit{psaA}, \textit{psaB}, and \textit{psaC}, have been described recently (12). Such mutants were incapable of \( \text{CO}_2 \) fixation or photoautotrophic growth, but reintroduction of the deleted gene restored photoautotrophic growth (13–15). Two hypotheses can be formulated to explain the discrepancy between the results with deletion mutants and \textit{trans}-splicing mutants. On the one hand, the B4 and F8 strains could have harbored undetected amounts of PS I that allowed synthesis of enough NADPH to fix \( \text{CO}_2 \) and grow photoautotrophically. On the other hand, the B4 and F8 strains could have possessed an as yet unidentified system that allowed PS I-independent reduction of NADP\(^+\). We have undertaken an analysis of several different PS I-deficient mutants (see Fig. 1) to examine critically the claim that PS I-deficient mutants can perform photosynthesis. We have used immunoblots to detect PS I subunits as well as spectroscopic means to measure photochemical activity in wild-type and mutant strains.
toxicidizable P_{700}, the primary electron donor of PS I (for reviews on PS I, see Refs. 16 and 17). We have made use of real time mass spectroscopy to measure the rates of O₂ photoevolution, CO₂ fixation, and H₂ photoevolution. Our results indicate very clearly that photosynthesis requires the presence of active PS I.

**EXPERIMENTAL PROCEDURES**

**Strains and Genetic Methods**—Our WT strain was isolated as an mt⁺ segregant from a cross between two derivatives of the 137c strain (18). The original deletions of psaA and psaC were made in this strain (12). Isolates of the B4 strain (10) were kindly provided by Dr. J. Girard Bascou (Institut de Biologie Physico-Chimique) and Dr. L. Mets (University of Chicago). The B4 strain (1) was kindly provided by Dr. L. Mets.

Bioballistic chloroplast transformations were performed with plasmids designed to delete the psaA and psaC genes, and homoplasmicity of these deletions was assessed by polymerase chain reactions (12). The plasmid pBR322 was used for all cloning procedures and was propagated in E. coli.(18).

**Growth Conditions, Fluorescence Induction, and Immunoblot Analysis**—Tris acetate/ phosphate medium (TAP) and HSM for heterotrophic and photoautotrophic growth, respectively, were prepared as described (18). Chloramydonaes reinhardtii cultures were maintained at 25 °C. In general, 100-ml TAP cultures were shaken at 180 rpm in 500-ml Erlenmeyer flasks, aerobic cultures were prepared as described (18). Chloramydonaes reinhardtii cultures were maintained at 25 °C. In general, 100-ml TAP cultures were shaken at 180 rpm in 500-ml Erlenmeyer flasks under low illumination (~1 μmol of photons m⁻² s⁻¹). For growth tests, 12 μl of log phase cultures were spotted onto agar media. TAP plates were kept under low light (~1 μmol of photons m⁻² s⁻¹). HSM plates were subjected to anaerobiosis using the Bioballistic chloroplast transformation systems, glucose (20 mM final concentration) and glucose oxidase (2 mg/ml final concentration) were added to verify that O₂ depletion before illumination was complete and that hydrogenase induction and activity were not affected by residual oxygen.

**RESULTS**

**Wild Type**—To introduce the methods used in this work, we will first describe characterization of the WT strain (see "Experimental Procedures"). This can grow photoautotrophically as well as heterotrophically on acetate-containing medium in the dark (Fig. 2A). Using specific antibodies against either PsxA (an integral membrane protein) or PsAD (a stromal subunit involved in docking ferredoxin to the acceptor side of PS I; Refs. 25 and 26), we could easily visualize these polypeptides in membranes prepared from the WT strain (Fig. 2A). The in vivo fluorescence induction kinetics (Fig. 2B) are typical of normal algae (27). The rise in fluorescence, which is correlated with reduced QA in PS II, is due to the reduction of the plastoquinone pool, while the subsequent drop has been interpreted as originating from oxidation of the plastoquinone pool due to the combined action of PS I and cytochrome bd (28).

In order to measure P₇₀₀ quantitatively, we monitored the kinetics of the increase and decrease of P₇₀₀ by following the increase of its absorbance at 832 nm (see "Experimental Procedures"). Photooxidation of P₇₀₀ results in a broad absorbance in the near-IR due to the loss of the ground state character in oxidized chlorophyll and is monitored as an increase in absorbance at 832 nm. Optimization experiments indicated that the maximum amount of P₇₀₀⁺ could be observed within 1 s of illumination with far-red light in the presence of sodium ascorbate and 2,6-dichlorophenolindophenol. The near-IR measuring beam is not able to photooxidize P₇₀₀ by
The O₂ evolution rate was equivalent to this value (Table I), as expected; for each molecule of O₂ produced, one electron was transferred from ferredoxin (31–33). After removal of O₂, production of H₂ in WT cells was observed immediately upon illumination (Fig. 5).

**TABLE I**

| Strain genotype | Amount of PSI* | P₇₀₀ | Phototrophic growth† | CO₂ uptake rate (light) | O₂ evolution rate (light) | Respiration rate (dark) | H₂ evolution rate (light) |
|----------------|---------------|------|----------------------|------------------------|--------------------------|------------------------|--------------------------|
| Nuclear        | Chloroplast   | PsaA | PsaD                 |                         |                          |                        |                          |
| WT             | WT            | 100  | 100                  | ++                     | 1500                     | 1550                   | 210                      | 300                      | 1200                    |
| WT             | psaAΔ         | 0    | 0                    | ++                     | <5                       | 210                    | 500                      | 690                      | <1                      |
| WT             | psaΔ         | 18    | 2                   | 1                      | <5                       | 210                    | 290                      | 485                      | 3                       |
| WT             | psaB-FUD26    | 57    | 52                   | 61                    | ++                      | 1000                   | 1070                     | 450                      | 600                     | 930                      |
| F₈             | WT            | 10    | 10                   | 8                      | +                        | 310                    | 330                      | 320                      | 320                     | 130                      |
| F₈             | psaΔ         | 2      | 0                    | 1                      | <5                       | 140                    | 400                      | 550                      | <1                      |
| F₈             | psaBΔ + psaA | 10    | 8                    | 4.3                  | +                        | 280                    | 350                      | 450                      | 330                     | 150                      |
| F₈             | psaΔ         | 1      | 0                    | 1                      | <5                       | 100                    | 310                      | 410                      | <1                      |
| B₄             | WT            | 7      | 0                   | 3.3                  |                         | <5                     | 130                      | 310                      | 420                     | <1                      |
| B₄             | psaΔ         | 4      | 20                   | 3.6                  |                         | <5                     | 120                      | 390                      | 540                     | <1                      |
| B₄-r₂          | WT            | 150   | 130                  | 39                  | ++                      | 1100                   | 1600                     | 320                      | 550                     | 1100                    |

* Levels of PsaA and PsaD polypeptides in total cellular membranes were determined by densitometry of the immunoblot shown in Fig. 1, which is representative of results seen in other such experiments (data not shown). Levels of photooxidizable P₇₀₀ were determined by comparing the signals of the various mutants with WT as shown in Fig. 2. Note that the disagreement between the detection of PS I by immunological or spectroscopic means may be due to the different ways used to normalize the signals (i.e., to equal amounts of protein or to equal numbers of cells).

† The phototrophic rates are expressed qualitatively (+++, normal growth; ++, suboptimal growth; +, poor growth; −, no growth).

‡ These values are expressed as nmol min⁻¹ (mg of chlorophyll)⁻¹. The "light" rates were recorded at an illumination of 300 μmol of photons m⁻² s⁻¹, which allows fully saturated (low level) O₂ photoevolution in PS I-deficient mutants and approximately 75% of the saturated value in WT cells. The lower limits of detection were 5 nmol of CO₂ min⁻¹ (mg of chlorophyll)⁻¹ and 1 nmol of H₂ min⁻¹ (mg of chlorophyll)⁻¹.

§ The mutants FUD26 and B₄-r₂ show photoinduced signals with slightly longer oxidation and reduction kinetics.

itself, thus eliminating a possibility of underestimating the amount of photochemically active P₇₀₀. The maximum level of P₇₀₀ oxidation is attained using far-red excitation, since this preferentially excites PS I, thereby decreasing the rate of P₇₀₀ reduction in the light. In WT cells, P₇₀₀ is oxidized within 200 ms of illumination and is completely re-reduced within 2 s after termination of excitation (Fig. 3).

We used continuous mass spectrometry to determine the *in vivo* rates of O₂ evolution, CO₂ fixation, and H₂ photoevolution. We were able to distinguish photosynthetic from respiratory activities by the inclusion of [¹⁸O]O₂ and [¹³C]CO₂ (25). The disappearance of [¹³C]CO₂ starting within 10 s after the commencement of illumination is indicative of the activation of the Calvin cycle enzymes, which then cooperate to convert gaseous CO₂ to carbohydrate (30). The WT cells removed CO₂ from the medium in a light-dependent manner at a steady-state rate of 1.5 μmol min⁻¹ mg of chlorophyll⁻¹ when illuminated with 300 μmol of photons m⁻² s⁻¹ (Fig. 4; Table I). When the photon flux was reduced 100-fold, CO₂ uptake occurred at 1.2% of this rate (Fig. 4). The O₂ evolution rate was equivalent to this value (Table I), as expected; for each molecule of O₂ produced, one molecule of CO₂ is fixed. The respiration rate, as measured by uptake of [¹⁸O]O₂, was not greatly affected by the light (Table I). Under anaerobic conditions, the chloroplast enzyme hydrogenase is induced and can serve as an alternate acceptor of electrons from ferredoxin (31–33). After removal of O₂, production of H₂ in WT cells was observed immediately upon illumination (Fig. 5).

**psaAΔ and psaΔ**—In order to examine mutants that should contain no PS I, we made use of mutants with deletions in the chloroplast genes *psaA*, *psaB*, or *psaC* (Ref. 12; see Fig. 1). These deletions remove the entire coding sequence of *psa* exon 3 (which encodes the last 661 amino acid residues of the 751-residue PsaA polypeptide), 96% of the *psaB* gene, or all of the *psaC* gene. Thus, such deletion mutants should be incapable of reversion.

We found that the *psaA* and *psaC* deletion mutants could not grow photoautotrophically and gave fluorescence induction patterns typical of mutants lacking PS I (Fig. 2; results were identical for *psaBΔ*, data not shown), in that the fluorescence rose to a high level and remained constant (34). Photoautotrophic growth can be restored to these strains by reintroduction of the deleted gene (14, 15). We could detect no PsaA or PsaD polypeptides in membranes from the *psaAΔ* strain (Fig. 2A). As has been observed previously (12, 34), the *psaCA* mutant could accumulate some PsaA polypeptide at roughly 5–10% of the WT value (Fig. 2A). However, membranes from this mutant contained no detectable PsaD polypeptide, indicating that the stromal side of PS I is significantly perturbed due to the lack of PsaC. No photooxidizable P₇₀₀ could be detected in either mutant (Fig. 3; Table I).

We have previously shown that the *psaBΔ* mutant cannot fix CO₂ (13). The *psaAΔ* and *psaCA* mutants were also incapable of CO₂ fixation (Fig. 4; Table I), and no significant H₂ photoevolution could be detected in the *psaAΔ* mutant (Fig. 5; Table I).

Significant O₂ photoevolution does occur in these mutants (Table I); however, in this case, the electrons appear to be directed toward respiration rather than CO₂ fixation or H₂ evolution. Note that the light respiration rates in these PS I-deficient mutants are essentially the sum of the dark respiration and O₂ evolution rates (Table I). Previous experiments (13) with the *psaΔ* mutant under varying light showed that it could evolve O₂ at a maximal rate about 4% of that of the maximal WT rate but that this evolved O₂ was almost quantitatively matched by an increase in the respiration rate (i.e., there is no net O₂ evolution).

**FUD26**—The FUD26 mutant was shown to be PS I-deficient due to a 4-base pair deletion in the *psaB* gene, causing a frameshift and truncation of the last 10 kDa of the PsaB polypeptide (Ref. 19; see Fig. 1). Thus, this mutant should be incapable of synthesizing active PS I. However, we found that the FUD26 isolate provided to us was able to grow photoautotrophically (Fig. 2A) and had a fluorescence induction pattern consistent with significant reoxidation of the plastoquinone pool (data not shown). We detected the PsaA and PsaD polypeptides in significant amounts in membranes from this strain (Fig. 2A). Moreover, we could detect photooxidizable P₇₀₀ at a level approximately 60% that of WT (Fig. 3). This isolate was able to fix CO₂ and photoevolve H₂ at rates 67 and 77% of WT, respectively (Table I).

We were surprised by the presence of PS I in this strain, considering the presence of a frameshift in the *psaB* gene. An isolate of FUD26 obtained from Dr. Girard-Bascou showed no growth on minimal medium, similar to the original description of this mutant (20). We hypothesize that the mutant has undergone some type of mutation event that allows it to synthe-
size some full-length PsaB. We note that Greenbaum et al. (35) estimated during their recent experiments that this mutant had levels of PS I equivalent to 11–14% of WT; it would thus appear that this mutant can revert to various levels of PsaB expression. These results highlight the danger of using mutants in which the genetic information specifying the protein of interest has not been completely obliterated.

F8—The F8 mutant has a nuclear mutation that prevents trans-splicing of the psaA mRNA (Refs. 10 and 11; see Fig. 1). The F8 strain was able to grow slowly on minimal medium (Fig. 2A) and exhibited a fluorescence induction pattern characteristic of mutants containing small amounts of PS I (Fig. 2B; Refs. 15, 36). Membranes from this strain contained small but detectable amounts of the PsaA and PsaD subunits. Using densitometry, we estimated that these polypeptides were present at roughly 10% of the WT amount when normalized to total membrane protein (note that PsaA and PsaD were present at the same stoichiometry as in the WT). Photooxidizable P700 in this strain represented roughly 8% of the WT level (Fig. 3; Table I), indicating that a small amount of active PS I was present in this strain. We observed light-dependent CO2 fixation at roughly 20% of the WT rate (Fig. 4; Table I) and H2 photoproduction at roughly 10% the WT rate (Fig. 5; Table I). An isolate of F8 from the Chlamydomonas Stock Center was similar to the one shown here in that it contained small amounts of PsaA and grew slowly on minimal medium (data not shown); thus, this phenotype might be a general phenomenon and not due to an isolated reversion event.

F8—Deletion and Reintroduction of psaA and Deletion of psaC—The F8 mutant provided us the opportunity to distinguish between the two hypotheses posed earlier by use of a genetic test. The third exon of psaA has been deleted in F8 in the same way as the psaAΔ mutant.

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**Fig. 1.** Graphic depiction of the strains used in this study. Each oval represents one strain; the upper circle is the nucleus, and the lower shape is the chloroplast. Within the chloroplast, the oval to the left is the chloroplast genome, and the double oval at the bottom represents the thylakoid membrane. WT, the mature psaA mRNA is assembled from three separate transcripts arising from the chloroplast genome. Translation of this message gives rise to the PsaA polypeptide (represented by the serpentine form), which forms half the core of PS I. psaAΔ, the third exon of psaA has been deleted in this strain by chloroplast transformation. Thus, there will be no PsaA polypeptide. FUD26, the chloroplastic psaB gene has a four-base pair deletion (*) in the FUD26 mutant, which results in a frameshift and a truncated gene product. B4, F8, these mutants each have a mutation (X) in the nuclear genome that blocks trans-splicing of the psaA mRNA. They have very little or no mature psaA mRNA, and consequently very little or no PsaA polypeptide. Gray forms and shadowed letters symbolize this indeterminate quantity. F8-psaAΔ, the third exon of psaA has been deleted in F8 in the same way as the psaAΔ mutant.
Fig. 2. A, growth tests and immunoblot analysis of the various mutants. The indicated cultures were grown to log phase in acetate-containing medium under low illumination and then spotted onto either acetate or minimal agar. The acetate plates were kept under low light (<1 μmol of photons m⁻² s⁻¹), while the minimal plates were subjected to anaerobic conditions and illuminated (300 μmol of photons m⁻² s⁻¹). Membrane proteins (75 μg/lane) prepared from the cultures were immunoblotted using a combination of anti-PsaA and anti-PsaD antibodies. The leftmost lane is a 5-fold dilution of the WT extract into the psaAΔ extract (thus, 20% of WT). Two independent transformants each are shown of the psaAΔ and psaCΔ deletion mutants of F8. B4-r2 is a photoautotrophic derivative of B4. Note the presence of a cross-reacting protein that is present in all extracts and that migrates slightly faster than PsaA. B, in vivo fluorescence induction kinetics. Log phase TAP cultures of the various mutants were illuminated with blue light (75 μmol of photons m⁻² s⁻¹), and the red chlorophyll fluorescence was measured using a laboratory-built video fluorometer. Strains shown are WT (●), psaAΔ (○), F8 (▲), F8-psaAΔ (△), F8-psaAΔ+psaA (●), B4 (□), and B4-r2 (■).

The absence of PS I, then ablation of psaA should have little effect. However, after deletion of psaA, the F8 strain lost the ability to grow photoautotrophically and displayed a fluorescence induction curve typical of a PS I-deficient mutant (Fig. 2). The small amount of PsaA and PsaD detectable in F8 disappeared after deletion of psaA (Fig. 2A), and photooxidizable P700 signals were no longer visible in the F8-psaAΔ strains (Fig. 3). Along with the loss of PS I upon deletion of psaA, F8 also lost the ability to fix CO2 (Fig. 4) and photoevolve H2 (Fig. 5).

We considered the possibility that transformation might cause an unforeseen effect, besides the loss of PS I, that could explain the lack of photosynthesis in the F8-psaAΔ mutants. The particle bombardment technique used to introduce DNA into the chloroplast might also insert DNA into the nucleus, thus creating random mutations. However, it seems unlikely that secondary nuclear mutations could explain the loss of photosynthesis, since we have examined several independent transformants. To further exclude this possibility, we reintroduced psaA exon 3 into two independently derived F8-psaAΔ strains and selected transformants by virtue of a co-integrated antibiotic resistance gene (see “Experimental Procedures”). We found that this restored to all of the transformants (referred to as “F8-psaAΔ+psaA”) both the slow photoautotrophic growth and the fluorescence induction curve seen previously with the original F8 mutant (data not shown; Fig. 2 shows results from one representative transformant from each of the two F8-psaAΔ strains). The levels of PsaA and PsaD polypeptides (Fig. 2A) as well as photooxidizable P700 (Fig. 3) were restored to their original low levels after reintroduction of psaA. Moreover, the CO2 fixation and H2 evolution activities returned to the same levels seen in F8 (Figs. 4 and 5; Table I).

Another unlikely possibility was that the DNA used to replace psaA was somehow affecting the expression of other gene(s) required for the hypothetical “PS I-independent photosynthesis” process. However, we have also deleted the gene for Psac in F8. These genes are distant from each other in the chloroplast genome (18); additionally, the piece of DNA used to delete psaC was different from that used to delete psaA and psaB (12). As in our WT strain, the loss of Psac in the F8 strain caused a loss of photoautotrophic growth (Fig. 2A). We could no longer detect PS I in the F8-psaCΔ transformants, either immunologically (Fig. 2A) or spectroscopically (Fig. 3). Additionally, neither CO2 fixation nor H2 photoevolution could be detected in the F8-psaCΔ derivatives (Table I). All of these results
I). Thus, we conclude that this strain has either no PS I or polypeptides in membranes (Fig. 2). The B4 mutant (Fig. 2) had a fluorescence induction pattern typical of a PS I-deficient mutant. We could not grow photoautotrophically (Fig. 2). We could not detect the PsaA or PsaD polypeptide contaminating the membrane preparations, nor could we observe any small photoinduced P700 signals, indicating that this signal is not related to P700. The kinetics of the small photoinduced signal at 832 nm are much faster in this mutant than in the other strains displaying related to P700.

are entirely consistent with the hypothesis that the photosynthetic activities occurring in F8 are dependent upon the small stromal PsaD polypeptide contaminating the membrane preparations, nor could we fix CO2 or photoevolve H2 (Table I). Since the B4 strain was incapable of CO2 fixation, we were not surprised that deletion of psaA in this strain had no effect. The B4-psaAD strain appeared identical to the original B4 strain; it was unable to grow photoautotrophically (Fig. 2A), it had no PsaA polypeptide (Fig. 2A), and F8-psaAD+psaA strains. C, B4 (●) and B4-r2 (○) strains.

Fig. 4. CO2 exchange measured during dark/light transitions in different mutants using mass spectrometry and 13C-enriched CO2. The light (300 μmol of photons m-2 s-1) was turned on when denoted by the vertical dotted line. Chlorophyll concentrations were 20 μg ml-1. A, WT (○, ▼), psaAD (○), and psaC (▼) mutants. WT CO2 consumption in low light (3 μmol of photons m-2 s-1) was 12% of the consumption rate at high light fluence (300 μmol of photons m-2 s-1), which allowed saturating oxygen evolution in all mutants and about 75% of the saturating oxygen evolution activity in WT. B, CO2 exchange during dark/light transitions in the B4 (○), F8 (○), F8-psaAD (▼), and F8-psaAD+psaA (▼) strains.

Fig. 5. Photoproduction of H2 during dark/light transitions measured by mass spectrometry. Cultures were first subjected to anaerobic conditions in order to induce hydrogenase. The light (300 μmol of photons m-2 s-1) was turned on when indicated by the arrow. Chlorophyll concentrations were 20 μg ml-1. A, WT (●) and psaAD (○) strains. B, F8 (●), F8-psaAD (○), and F8-psaAD+psaA strains. C, B4 (●) and B4-r2 (○) strains.

very small amounts. Using mass spectrometry, we could not detect light-dependent uptake of CO2 (Fig. 4) or production of H2 (Fig. 5).

Since the B4 strain was incapable of CO2 fixation, we were not surprised that deletion of psaA in this strain had no effect. The B4-psaAD strain appeared identical to the original B4 strain; it was unable to grow photoautotrophically (Fig. 2A), it had no PsaA polypeptide (Fig. 2A; note that detection of some PsaD in this experiment is not reproducible and may represent some stromal PsaD polypeptide contaminating the membrane preparations), nor could it fix CO2 or photoevolve H2 (Table I). The kinetics of the small photoinduced signal at 832 nm are much faster in this mutant than in the other strains displaying photoinduced P700 signals, indicating that this signal is not related to P700.

Given these facts, it is difficult to explain the reported fixation of CO2 and photoevolution of H2 in the B4 strain (1, 2). We hypothesize that the B4 mutant can phenotypically revert. We isolated two photoautotrophic revertants from B4, and they both expressed PsaA (13). Fortuitously, we managed to isolate a PsaA-expressing photosynthetic revertant, B4-r2, from the transformant that gave rise to B4-psaA3 before all copies of the psaA gene had been removed (see “Experimental Procedures”). The B4-r2 strain was essentially the same as WT; it expressed PsaA at a high level (Fig. 2A), showed a normal fluorescence induction pattern (Fig. 2B), and contained photooxidizable P700 (Fig. 3) at roughly 40% of the WT level (Table I), and could fix CO2 and evolve H2 at normal rates (Fig. 4; Table I). However, after the deletion had become homoplasmic, giving rise to the B4-psaA strain, we could not obtain such photosynthetic revertants (see “Experimental Procedures”). Taken together, it
would seem that the only way for B4 to revert to a photosynthetically competent state is to express PsaA. We have performed the complementary experiment by transformation with a construct that replaces psaA exon 3 with a "prespliced" psaA gene, thus bypassing the trans-splicing defect in B4. Introduction of this gene into either B4 or B4- psaA confers photoautotrophic growth to the transformants. Thus, photosynthesis in B4 also correlates with the presence of PS I.

**DISCUSSION**

The Z-scheme of linear electron transport explains a large body of data. Exceptions to the pathway of linear transport are well known and include cyclic electron transport around PS I and cytochrome b6f, injection of electrons into the system by hydrogenase, the facultative usage of sulfate as an alternate electron source in certain cyanobacteria (37–39), and the use of oxygen as an alternate electron acceptor (13, 29). Be that as it may, the thermodynamic underpinnings of the Z-scheme appear to rest on solid ground. PS II is a strong oxidant (midpoint redox potential \( E_m \) of \( P_{680}^+ = +1100 \) mV) that is capable of oxidizing water (\( E_m = +820 \) mV) but is a relatively poor reductant, able to reduce plastoquinone \( (E_m = +100 \) mV) but not ferredoxin \( (E_m = -430 \) mV) or NADP\(^+ \) \( (E_m = -320 \) mV). PS I is a strong reductant, whose iron-sulfur clusters \( (E_m = -580 \) mV) are capable of reducing ferredoxin but whose oxidized primary donor \( (E_m = +450 \) mV) is incapable of oxidizing water.

However, the discovery that pheophytin (\( E_m = -610 \) mV) is the primary electron acceptor within PS II (40, 41) made feasible the idea that PS II could reduce ferredoxin or NADP\(^+ \). This coupled with the fact that PS II and PS I are differentially localized to granal and stromal thylakoid membranes, respectively (42), prompted the proposal that PS II in the granal membrane could reduce ferredoxin, while PS I in the stromal membrane was involved in cyclic transport for the generation of ATP. Albertsson et al. (43) observed reduction of NADP\(^+ \) coupled with \( H_2 \) oxidation in vesicles enriched in PS II, and Arnon and Barber (44) presented evidence that isolated PS II reaction centers could reduce ferredoxin. The involvement of residual PS I in these reactions was suggested by the fact that both required plastocyanin, a well known PS I electron donor and that the first could be blocked by a cytochrome b6f inhibitor. While PS I-dependent reactions can be studied in the absence of PS II due to the existence of specific inhibitors of PS II and the ability to independently excite PS I using far-red illumination, the study of PS I-dependent reactions in the absence of PS I poses more difficulties. The use of genetic mutants provides a novel way to overcome this problem.

The pioneering genetic and biophysical work of Levine and others (45, 46) in their analysis of various \( C. reinhardtii \) photosynthetic mutants provided the framework of the Z-scheme. Many mutants specifically defective in PS I are available (e.g. Refs. 10 and 11). The psaA gene is split into 3 exons that are widely separated and located on different strands of the \( C. reinhardtii \) chloroplast genome (47). These exons are separately transcribed and then joined together in a process known as "trans-splicing" (48), which regulates the number of the genes necessary for PS I biosynthesis (11). The B4 and F8 mutants are defective in the splicing of the first and second exons (1, 10, 11).

In general, PS I-deficient mutants have been reported to be nonphotoautotrophic (18), but it was possible that this phenotype was due to pleiotropic mutations that caused the coordinate but unrelated loss of PS I and photosynthetic activity. A reverse genetics approach would eliminate this possibility, as one could eliminate a gene for a specific PS I subunit without any expectation as to the phenotypic consequences. The \( psaA \), \( psaB \), and \( psaC \) genes have been deleted from the chloroplast genome in \( C. reinhardtii \) (12, 49), and these deletion mutants were nonphotosynthetic. PS I subunit genes have also been deleted in two cyanobacterial species with attendant loss of photoautotrophic growth (50, 51). Thus, the "forward" and "reverse" genetic approaches agree that PS I is required for photoautotrophic growth.

We have reported previously that the PS mutant ceased to grow photoautotrophically after deletion of \( psaA \) exon 3 and that the B4 mutant was nonphotoautotrophic (13). Here we extend that work. By spectroscopic observation of \( P_{700} \) photooxidation, we demonstrated that active PS I exists in all strains capable of photoautotrophic growth. We have eliminated the unlikely possibility that the loss of photosynthesis in \( psaA \) deletion mutants was due to unrelated genetic damage by reintroducing \( psaA \) into them and observing the restoration of \( CO_2 \) fixation. Photoautotrophic growth and \( CO_2 \) fixation were also lost after deletion of the \( psaC \) gene. This indicates that the presence or absence of these capabilities correlates only with the presence of PS I and not with the specific method used to eliminate it. In this regard, the lack of \( H_2 \) evolution in PS I deletion mutants is of special note. \( H_2 \) photoevolution was undetectable in mutants with deletions of \( psaA \), \( psaB \), or \( psaC \), no matter what their genetic background. While \( CO_2 \) fixation requires both NADPH and ATP, \( H_2 \) production requires only reduced ferredoxin and is thus a more sensitive test for reduction of ferredoxin in vivo. The lack of \( H_2 \) photoevolution in PS I-deficient strains is therefore a strong indication that ferredoxin is not photooxidized in the absence of PS I.

Using the sensitive technique of mass spectrometry combined with isotopic labeling, we have shown that PS I deletion mutants are incapable of fixing inorganic carbon. However, we found that all PS I-deficient cells were capable of sustained water photooxidation, although the rate could vary between strains (Table I). We have previously observed such \( O_2 \) photooxidation in mutants lacking PS I (13, 29), and it can represent a rate approximately 5–10% of that seen in WT cells. This activity reflects the ability of the plastid to reoxidize the plastoquinone pool independently of the cytochrome b6f complex and PS I. Since the respiration rate was seen to increase by an approximately equivalent amount, molecular oxygen is likely to serve as the ultimate electron acceptor.

Because the kinetics of electron transfer from the primary donor to the terminal acceptor are faster in PS I than in PS II, PS I should be able to handle the electron flow from a larger quantity of PS II (52). In fact, it has been proposed that the physical separation of the photosystems is required for efficient linear electron flow due to the much faster trapping kinetics of PS I (53). Whether or not the small amount of PS I in "leaky PS I mutants" could account for the observed amounts of photoevolved \( H_2 \) is still under discussion (35, 52). Although our work here does not directly address this issue, we observed rates of \( H_2 \) photoevolution that seem compatible with the amount of detectable PS I (Table I). Under normal conditions, the rate-limiting step of linear electron transport is at the level of the cytochrome b6f complex (54), and not at PS I. Therefore, one would expect that in mutants with low levels of PS I, the relative \( CO_2 \) uptake rates should be somewhat higher than the relative amounts of PS I. While we cannot absolutely exclude the possibility that PS I-independent and PS I-dependent electron transfer pathways from \( H_2 O \) to ferredoxin could exist side-by-side in mutants with low amounts of PS I, we cannot explain why this hypothetical PS I-independent electron flow would not occur in mutants lacking PS I. Boichenko (55) has recently measured the action spectrum of \( H_2 \) photoevolution in F8 cells and found that it is remarkably similar to that of PS I.

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2 C. Rivier, K. Redding, and J.-D. Rochaix, unpublished results.
and significantly different from the action spectrum of O₂ evolution, which resembles that of PS II. Our data are complementary to that of Boichenko, and we conclude likewise that the observed CO₂ fixation and H₂ photoevolution activities in the B4 and F8 strains are due to PS I.

CONCLUSION

We find that the in vivo capabilities of photoautotrophic growth, CO₂ fixation, and H₂ evolution require the presence of active PS I. We have found no case in which a mutant lacking detectable PS I could perform these functions. Thus, we disagree fundamentally with the conclusions arrived at by Greenbaum, Lee, and colleagues (1, 2). Furthermore, our results indicate that one must be very careful in the choice of mutant to study; mutants defective in expression can be suppressed and small deletions in genes can be repaired, but large deletions of entire open reading frames encoding key structural components cannot revert or result in leaky phenotypes.

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