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Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilizes photosystem I

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The chloroplast gene *psaC* encoding the iron sulfur protein of photosystem I (PSI) from the green alga *Chlamydomonas reinhardtii* has been cloned and characterized. The deduced amino acid sequence is highly related to that of higher plants and cyanobacteria. Using a particle gun, wild type *C.reinhardtii* cells have been transformed with a plasmid carrying the *psaC* gene disrupted by an *aadA* gene cassette designed to express spectinomycin/streptomycin resistance in the chloroplast. Transformants selected on plates containing acetate as a reduced carbon source and spectinomycin are unable to grow on minimal medium lacking acetate and are deficient in PSI activity. Southern blot analysis of total cell DNA of the transformants shows that the wild type *psaC* gene has been replaced by the interrupted *psaC* gene through homologous recombination. While authentic transcripts of the *psaC* gene are no longer detected, *aadA* gives rise to a few transcripts in the transformants. Biochemical analysis indicates that neither PSI reaction center subunits nor the seven small subunits belonging to PSI accumulate stably in the thylakoid membranes of the transformants. Pulse-chase labeling of cell proteins shows that the PSI reaction center subunits are synthesized normally but turn over rapidly in the transformants. We conclude that the iron sulfur binding protein encoded by the *psaC* gene is an essential component, both for photochemical activity and for stable assembly of PSI. The present study suggests that any chloroplast gene encoding a component of the photosynthetic apparatus can be disrupted in *C.reinhardtii* using the strategy described.

Key words: Chlamydomonas reinhardtii/chloroplast transformation/photosystem I/*psaC* gene

Introduction

Photosystem I (PSI) is a multiprotein complex and drives electron transport from plastocyanin to ferredoxin using light energy. This complex is embedded in the thylakoid membranes and consists of two large homologous subunits with apparent molecular mass of 60-70 kd encoded by the chloroplast genes *psaA* and *psaB* and several small subunits encoded by the nuclear genes *psaD*, *psaE*, *psaF*, *psaG*, *psaH* and *psaK* (see Golbeck and Byrant, 1991 for review). Recently, three additional small subunits of PSI encoded by the chloroplast genes *psaC* (Hayashida *et al.*, 1987; Høj *et al.*, 1987; Oh-oka *et al.*, 1987), *psaI* (Scheller *et al.*, 1989a) and *psaJ* (Koike *et al.*, 1989) have been found. The primary donor chlorophyll, P700, two intermediate acceptors, A0 and A1, and the iron sulfur center X, as well as core antenna pigment molecules have been shown to be bound to the heterodimer of the two large subunits (Golbeck and Cornelius, 1986). The iron sulfur centers A and B which function as terminal electron acceptors of PSI are believed to be present on the 8-9 kd polypeptide, also designated as subunit VII in higher plants, encoded by *psaC* (Hayashida *et al.*, 1987; Høj *et al.*, 1987; Oh-oka *et al.*, 1987). This protein has been isolated with cofactors and was shown to give rise to characteristic electron spin resonance signals when chemically reduced (Oh-oka *et al.*, 1988). In addition, reconstitution of this holocomplex with PSI core complex consisting of only *psaA* and *psaB* products was shown to restore electron transport activity from P700 to centers A and B (Golbeck *et al.*, 1988). One of the small subunits encoded by *psaD* (also designated as subunit II in higher plants or P20 in *Chlamydomonas reinhardtii*) is assumed to be located on the stromal side of the thylakoid membrane and to mediate binding of ferredoxin to the PSI reaction center complex (Zanetti and Merati, 1987). Another polypeptide encoded by *psaF* (also designated as subunit III in higher plants or P21 in *C.reinhardtii*) assumed to be present on the luminal side of the thylakoid membranes appears to form a docking site for plastocyanin (Bengis and Nelson, 1977; Wynn and Malkin, 1988; Hippler *et al.*, 1989). The roles of the other small subunits are not clear. PSI assembly thus appears to be complex because it involves the integration of more than 10 subunits and of a number of chlorophyll, phylloquinone and carotenoid molecules as well as iron sulfur clusters.

A very promising approach for studying the roles of the constituent polypeptides of PSI and for investigating the mechanism of assembly of this complex is to use directed mutagenesis. This method has already provided important insights into the function of photosystem II polypeptides in the transformable cyanobacteria (Vermaas *et al.*, 1986; Pakrasi *et al.*, 1988; Burnap and Sherman, 1991). However, since these organisms grow poorly in the absence of active PSI, the directed mutagenesis of PSI is limited (Chitnis *et al.*, 1989a,b). The green unicellular alga, *C.reinhardtii*, is able to grow heterotrophically in the presence of a reduced carbon source such as acetate, and numerous mutants deficient in PSI activity have been isolated (Chua *et al.*, 1975; Bennoun *et al.*, 1977; Girard *et al.*, 1980; Girard-Bascou, 1987). The chloroplast genes *psaA* and *psaB* (Kück *et al.*, 1987) as well as cDNA clones of *psaE*, *psaF*, *psaG*, *psaH* and *psaK* (Franzén *et al.*, 1989a,b) have been sequenced in *C.reinhardtii*. In addition, *C.reinhardtii* is amenable to both genetic and biochemical analyses.

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Successful transformation of the chloroplast of *C. reinhardtii* was achieved with a particle gun (Boynton *et al.*, 1988; Blowers *et al.*, 1989). In these studies, several chloroplast photosynthetic mutations were used for complementation by bombarding cells with appropriate wild type chloroplast DNA fragments and by selecting for growth on minimal medium lacking a reduced carbon source. More recently, Przibilla *et al.* (1981) have been able to introduce site specific mutations in the *psbA* gene of *C.reinhardtii* by using herbicide resistance for the selection of the transformants. Non-photosynthetic selective markers are required, however, for the direct mutagenesis of chloroplast genes. We have therefore used the bacterial *aadA* gene conferring resistance to spectinomycin/streptomycin as a selective marker (M.Goldschmidt-Clermont, unpublished results). This gene was fused to chloroplast promoter and termination sequences to allow for its expression in the chloroplast of *C.reinhardtii*.

Amongst the numerous PSI mutants isolated, many appear to be affected in the expression of *psaA* and *psaB* that encode the two reaction center polypeptides of the complex. However none of the PSI mutants has been shown to be deficient in *psaC*. To study the role of the *psaC* product in PSI function and assembly, we have disrupted its chloroplast gene in *C.reinhardtii* with the *aadA* expression cassette. We report that the two reaction center polypeptides encoded by *psaA* and *psaB* are destabilized in the transformants as a result of the absence of the *psaC* product. This work represents the first description of a directed inactivation of a chloroplast gene and opens the door for chloroplast reverse genetics.

Results

Isolation and nucleotide sequence of the psaC gene

A 42mer oligonucleotide from the *psaC* gene of tobacco (Hayashida *et al.*, 1987) was used as a probe to map a homologous sequence on the chloroplast DNA of *C. reinhardtii*. Southern blot analysis showed that a single hybridizing sequence is present on a 10 kb *Eco*RI fragment (R23) and on a 37 kb *Bam*HI fragment (Ba15). The hybridizing sequence was located on a 1.05 kb *Sau*3A fragment of R23 (fragment X, see Figure 1) which was subcloned. The sequence of 420 bases from one end of the *Sau*3A fragment was determined and is shown in Figure 2. It reveals the presence of an open reading frame, encoding 81 amino acids, that is highly related to the published sequences of *psaC*; the corresponding protein has 86–89% homology with those of higher plants and cyanobacteria (see Table I). A striking feature is the presence of a pair of characteristic elements consisting of four cysteine residues, Cys-X-X-Cys-X-X-Cys-X-X-X-Cys-Pro (positions 11–22 and 48–59), which are assumed to be responsible for binding the iron sulfur centers A and B (Hayashida *et al.*, 1987; Høj *et al.*, 1987; Oh-oka *et al.*, 1987). Comparison of the primary sequence of the *psaC* product of *C.reinhardtii* with the corresponding sequence of other photosynthetic organisms reveals that the algal sequence differs at two positions, M39 and S73, from the consensus sequence derived from higher plants and cyanobacteria (I39 and T73).

Insertional inactivation of the psaC gene

Figure 1 shows a restriction map of the 10 kb *Eco*RI fragment (R23) carrying the *psaC* gene. To disrupt the *psaC* gene, a 4.6 kb *Pst*I–*Sal*I fragment carrying the *psaC* gene from R23 was subcloned into the Bluescript plasmid. The unique *Nco*I restriction site within the *psaC* gene (see Figure 1) was used to insert in either orientation a cassette designed to express spectinomycin/streptomycin resistance. This cassette consists of the bacterial *aadA* gene fused to a 0.65 kb promoter region of *atpA* and to a 0.45 kb downstream sequence of *rbcL* from *C.reinhardtii* and is thus designed to express spectinomycin/streptomycin resistance in the chloroplast of *C.reinhardtii* and to be used as a selective marker for transformation (M.Goldschmidt-Clermont, unpublished results).

Wild type cells were bombarded with the two constructs carrying the disrupted *psaC* gene. Cells were then replated on TAP plates containing spectinomycin to select for transformants. The fluorescence transients of dark adapted transformed cells were examined to test for PSI activity. Transformants with all *psaC* genes inactivated are expected to lack PSI activity because the *psaC* product is required for PSI function. Although most transformants tested in the present study displayed the fluorescence transients characteristic of PSI deficient mutants (Chua *et al.*, 1975; Bennoun *et al.*, 1977), some of them still showed a small amount of residual PSI activity. Four transformants which lacked PSI activity completely were plated three times consecutively on new spectinomycin plates. In the transformants 1B, 1D and 2B, the *aadA* gene is oriented in the opposite orientation to *psaC* whereas in 4B the orientations of both genes are the same. To test for stability, two colonies of each of these transformants were propagated separately as 1B1 and 1B2, 1D1 and 1D2, 2B1 and 2B2, 4B1 and 4B2, respectively.

Phenotype of the transformants

Figure 3 shows the fluorescence induction kinetics of the dark adapted cells of the wild type and of one transformant, 4B2. This transformant had a high fluorescence yield and displayed no slow fluorescence decrease ascribable to the photo-oxidation of PSII by PSI as already reported for both nuclear and chloroplast mutants deficient in PSI activity (Chua *et al.*, 1975; Bennoun *et al.*, 1977). The other transformants displayed similar fluorescence induction curves (data not shown). In addition, measurement of the light induced oxygen uptake activity in the presence of DCMU and KCN confirmed the complete loss of PSI activity in the transformants.

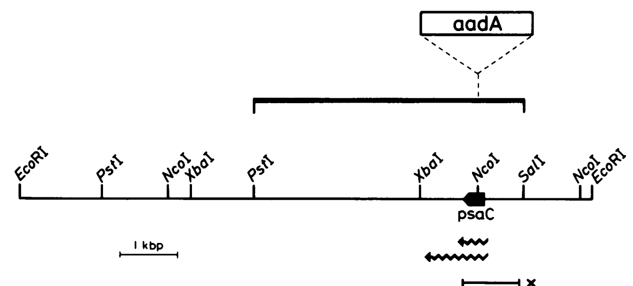

Fig. 1. Restriction map of chloroplast *Eco*RI fragment R23 containing the *psaC* gene from *C.reinhardtii*. For insertional inactivation of *psaC*, a 1.9 kb *aadA* cassette was inserted at the unique *Nco*I site in the 4.6 kb *Pst*I–*Sal*I fragment (shown at the top of the figure). The thick arrow shows the location of *psaC*. The two *psaC* transcripts are indicated by arrows. Fragment X shows a 1.05 kb *Sau*3A fragment used as probe for *psaC*.

Analysis of psaC DNA and RNA in the transformants

Total DNA from four transformants and from wild type was extracted and digested with *Xba*I and *Eco*RI. Hybridizations of the digests with a *psaC* probe (fragment X, see Figure 1) indicated that all the transformants had lost the 3.0 kb fragment corresponding to the wild type *Xba*I–*Eco*RI band

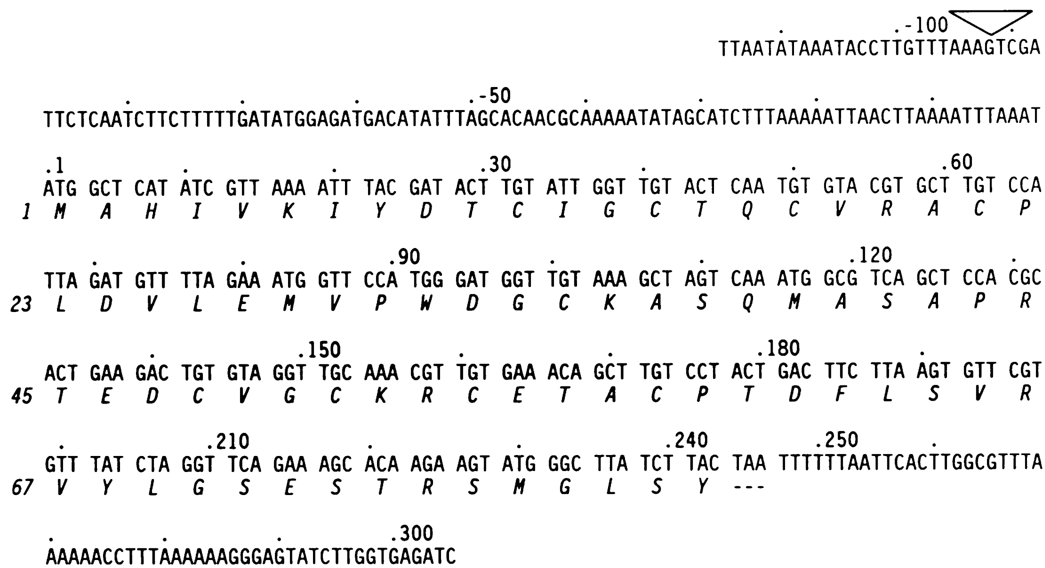

Fig. 2. Nucleotide sequence of the chloroplast *psaC* gene and its flanking regions in *C.reinhardtii*. The deduced amino acid sequence is indicated under the nucleotide sequence. The approximate position of the 5′ end of the *psaC* transcripts is indicated by an arrowhead.

Table I. Comparison of the amino acid sequences of the *psaC* gene products

									10										20										30										40	
	*			*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*		*	*	*	*	*		*	*	*	*	*		*		*		*	*
C.reinhardtii	A	H	I	V	K	I	Y	D	T	C	I	G	C	T	Q	C	V	R	A	C	P	L	D	V	L	E	M	V	P	W	D	G	C	K	A	S	Q	M	A	S
Barley	S		S																			T						I								K		I		
Wheat	S		S																			T						I								K		I		
Rice	S		S																			T						I								K		I		
Maize	S		S												H							T						I								K		I		
Spinach	S		S																			T						I								K		I		
Tobacco	S		S																			T						I								K		I		
Pea	S		S																			T						I								K		I		
Liverwort			A																			T						I								N		I		
C.paradoxa			T																			T												R		N		I		
Synechocystis	S		S																			T						I								K		I		
Synechococcus	S		S																																	G		I		
Nostoc	S		T																			T														A		I		

									50										60										70										80	
		*	*	*	*	*	*	*	*	*	*	*	*	*			*	*	*	*	*	*	*		*	*	*	*			*		*	*	*	*		*		*
C.reinhardtii	A	P	R	T	E	D	C	V	G	C	K	R	C	E	T	A	C	P	T	D	F	L	S	V	R	V	Y	L	G	S	E	S	T	R	S	M	G	L	S	Y
Barley															S															P		T					A			
Wheat															S															P		T					A			
Rice															S															P		T					A			
Maize															S															P		T					A			
Spinach															S														W	H		T							G	
Tobacco															S														W	H		T							A	
Pea															S														W	H		T							A	
Liverwort															S	R														N		T								
C.paradoxa															S									I						A		T								
Synechocystis															S														W	H		T							A	
Synechococcus	S																							I						A		T							A	
Nostoc	S																							I						A		T							A	

*Consensus sequence. Initiation methonine residue was omitted.

Sources of sequences: barley, Scheller *et al.* (1989)b; wheat and pea, Dunn and Gray, (1988); rice, Hiratsuka *et al.* (1989); maize, Schantz and Bogorad (1988); spinach, Oh-oka *et al.* (1988), Steppuhn *et al.* (1989); tobacco, Shinozaki *et al.* (1986); liverwort, Ohyama *et al.* (1986); *Cyanophora paradoxa*, Bryant *et al.* (1990); *Synechocystis* sp. PCC 6803, Anderson and McIntosh (1989) (cited by Golbeck and Bryant, 1991); *Synechococcus* sp. PCC 7002 and *Nostoc* sp. PCC 8009, Bryant *et al.* (1989).

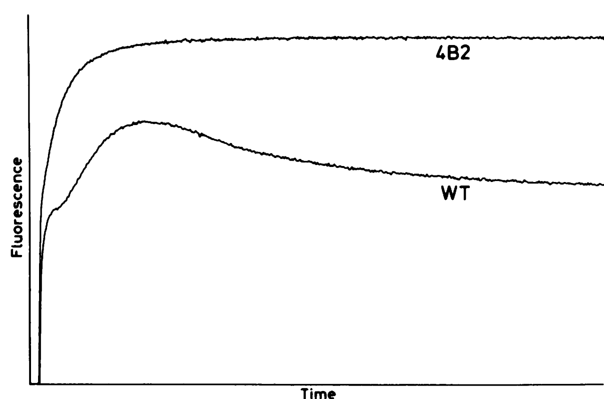

Fig. 3. Fluorescence induction kinetics of dark adapted cells of wild type and of transformant 4B2. Cells were grown on TAP agar plates and dark adapted for 10 min at room temperature before the measurement. Full time scale is 2 s.

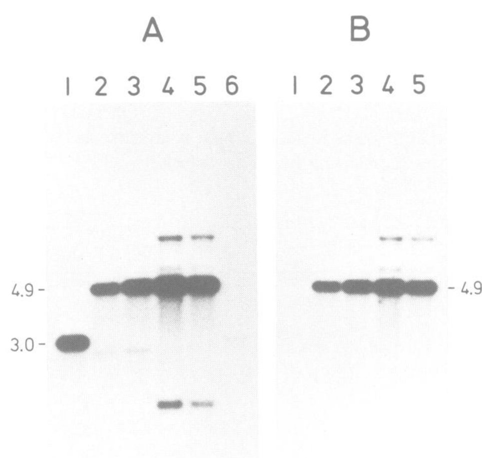

Fig. 4. Southern blot analysis of the total DNA from wild type and transformants. Total DNA from wild type and transformants was digested with *Eco*RI and *Xba*I, electrophoresed on a 0.6% agarose gel, transferred to nitrocellulose membrane, and hybridized with probe X specific for *psaC* (see Figure 1) (**A**), or an *aadA* probe (**B**). 1, wild type; 2, 1B1; 3, 1B2; 4, 4B1; 5, 4B2; 6, wild type; the amount of DNA is 1/80 of that in lane 1. The estimated sizes in kb are indicated.

which was replaced instead by a 4.9 kb fragment (Figure 4A). This size is expected if the *psaC* gene has been disrupted by the 1.9 kb *aadA* cassette (Figure 1). The identity of the 4.9 kb band was verified by hybridizing the same blot with a probe specific for *aadA*. As expected this probe hybridized only to the 4.9 kb fragment of the transformants and not to wild type DNA (Figure 4B). Although *C.reinhardtii* has a single chloroplast, the chloroplast DNA is a polyploid and is estimated to be present in ~70 copies per cell (Sueoka *et al.*, 1967). Since a single copy of chloroplast DNA can be detected under the conditions used as shown in Figure 4A, lane 6, we conclude that all chloroplast genomes in the transformants contain the transforming DNA. In addition,

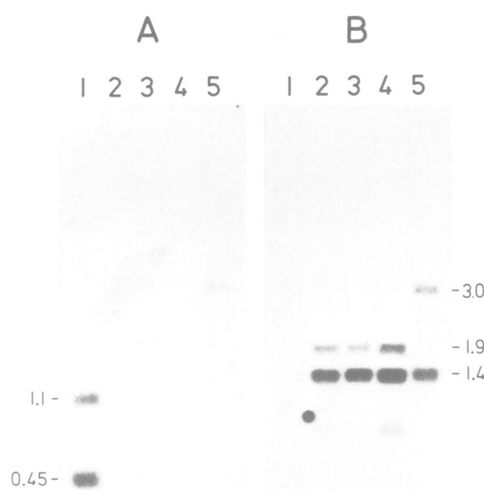

Fig. 5. Northern analysis of wild type and four transformants. Total RNA was fractionated on a denaturing 1.2% agarose gel, blotted onto Hybond-N membrane and hybridized with a probe specific for *psaC* (**A**) or *aadA* (**B**). 1, wild type; 2, 1B2; 3, 2B2; 4, 1D2; 5, 4B2. The estimated sizes are indicated in kb.

a few weak bands were also detected in the transformants 1B and 4B.

To determine the origin of these bands, transformants 1B (mt^+) was crossed to wild type (mt^-) and the DNA of the members of two complete tetrads were isolated and hybridized with the *psaC* probe. The major 4.9 kb band was transmitted to all the offspring as expected for a chloroplast DNA fragment (data not shown). Surprisingly, however, the faint bands were also inherited by all the offspring indicating that they, too, are of chloroplast origin (data not shown). It appears that part of the cassette is integrated elsewhere in all genomes or that the transforming DNA has integrated elsewhere in the chloroplast genome in a small portion of the chloroplast DNA molecules (<5%). Since the *aadA* expression cassette contains 0.65 kb from the *atpA* promoter and 0.45 kb of the *rbcL* terminator, it is possible that the transforming DNA may integrate in some cases in these regions of the chloroplast genome.

RNA was isolated from the transformants and the wild type, fractionated by gel electrophoresis under denaturing conditions and hybridized with the *psaC* (Figure 5A) and *aadA* (Figure 5B) probes. The *psaC* gene gives rise to two transcripts of 0.45 and 1.1 kb in the wild type. S1 mapping and primer extension assays showed that both transcripts have a common 5′ end which is located ~90 nucleotides upstream of the 5′ end of *psaC* (Figure 1). The 3′ ends of the short and long transcripts appear to map 120 and 750 nucleotides downstream from the 3′ end of *psaC* (data not shown). These transcripts are absent in the transformants as expected for a disrupted *psaC* gene. Instead, a larger transcript (3.0 kb) can be seen in transformant 4B2 where *aadA* has the same orientation as *psaC* (Figure 5A, lane 5). A transcript of the same size is also detected with the *aadA* probe, suggesting that this RNA results from the transcription

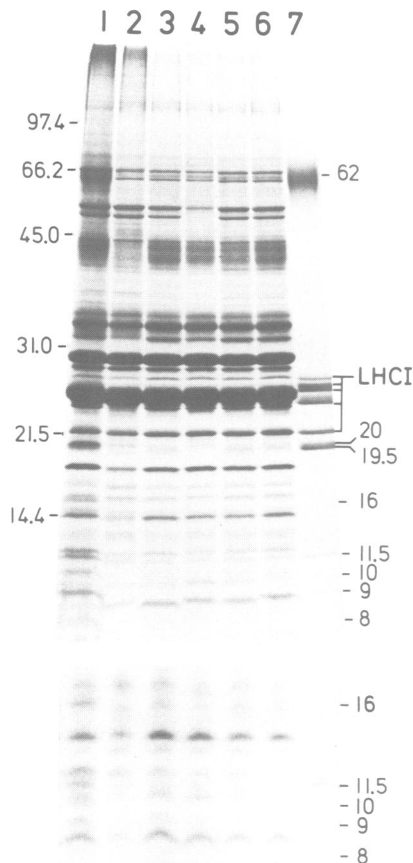

Fig. 6. Polypeptide profile analysis by SDS−PAGE. Thylakoid membranes (15 μg chlorophyll) of 1, wild type; 2, H13; 3, 1B2; 4, 2B2; 5, 1D2; 6, 4B2; 7, the purified PSI preparations from wild type (14 μ chlorophyll). Upper panel, 15−22.5% SDS−polyacrylamide gel according to Fling and Gregerson (1986); lower panel, 20% SDS−polyacrylamide gel according to Schägger and Jagow (1987). The gel was stained with Coomassie brilliant blue R250. LHCI represents the apoproteins of light harvesting chlorophyll *a/b* complex I (Wollman and Bennoun, 1982). The sizes of molecular markers are indicated to the left in kd. The estimated sizes of PSI polypeptides are indicated to the right in kd.

of the disrupted *psaC* gene (Figure 5B, lane 5). All transformants produce a common 1.4 kb transcript of *aadA*. An additional 1.9 kb *aadA* transcript is seen in the transformants 1B2, 2B2 and 1D2 where *aadA* has the opposite orientation to *psaC* (Figure 5B, lanes 2, 3 and 4).

Absence of the psaC product destabilizes the PSI complex

The PSI reaction center complex is a multiprotein complex and consists of the two large homologous reaction center proteins with apparent molecular masses of 62−70 kd encoded by the chloroplast *psaA* and *psaB* genes and of several small polypeptides. The polypeptide analysis of a number of nuclear and chloroplast mutants deficient in PSI from *C.reinhardtii* has shown that all these mutants lack a specific set of PSI polypeptides (Girard *et al.*, 1980; Girard-Bascou *et al.*, 1987). To test whether the PSI complex is present in the transformants, SDS−PAGE of thylakoid membranes was performed. In Figure 6, the polypeptide profiles of the thylakoid membranes of the wild type (lane 1), a stable PSI deficient mutant (lane 2), four independently isolated transformants (lanes 3, 4, 5, 6) and the purified wild type PSI reaction center (lane 7) are compared. The reaction center proteins that are seen as a diffuse band around 62 kd are not detected in the transformants. In addition, seven small polypeptides with molecular masses ranging from 20 to 8 kd are absent in the transformants. It can be seen that these seven polypeptides are also absent in the mutant, H13, but are all present in the purified PSI preparation. The 9 kd polypeptide corresponds most probably to the *psaC* product (see below). The other six polypeptides appear to correspond to the nuclear encoded polypeptides, P20, P21, P28, P30, P35 and P37. These results thus indicate that the transformants isolated in the present study have the same phenotype as the PSI deficient mutants reported previously that lack both PSI activity and that are missing the PSI polypeptide (Girard *et al.*, 1980). The low chlorophyll *a/b* ratio in the thylakoid membranes of the transformants (1.8) compared with that of wild type (2.1) fully agrees with the observation that the transformants lack the PSI reaction center complex which binds mainly chlorophyll *a*. Analysis of the chlorophyll protein complex of the thylakoid membranes by non-denaturing SDS−PAGE at 4°C confirmed the absence of CPI (P700 chlorophyll *a*-protein complex) in the transformants (data not shown). The light-harvesting chlorophyll *a/b* complex of PSI (LHC-I) is closely associated with the PSI complex as well and thus is copurified with the PSI reaction center preparation as shown in Figure 6, lane 7. However, the apoproteins of LHC-I accumulate normally in the thylakoid membranes of the transformants and of H13.

These results were confirmed by immunological means. Thylakoid proteins were fractionated by PAGE, electroblotted onto nitrocellulose membranes and reacted with antibodies against CPI (containing the two large PSI reaction center polypeptides) and P21 (corresponding to the *psaF* product). It can be seen in Figure 7A that the levels of the reaction center polypeptides are vastly reduced in the transformants and vary slightly from one transformant to the other (lanes 3−6). Based on experiments with diluted wild type samples we estimate that the proteins in the transformants accumulate to $<10\%$ of the wild type. Similarly, incubation of these protein blots with an antibody directed against P21 revealed that this PSI subunit does not accumulate in the transformants (Figure 7B). As a comparison, a PSI chloroplast mutant H13 which is deficient in *psaA* expression (Goldschmidt-Clermont *et al.*, 1990) does not accumulate a detectable amount of these proteins (Figure 7, lane 2).

The results obtained with the transformants suggest that the absence of the *psaC* product leads to the destabilization of the PSI complex. However it cannot be excluded that the *psaC* product could influence directly or indirectly the synthesis of other chloroplast encoded subunits, e.g. the two reaction center polypeptides. To test this possibility, cells from the transformants, wild type and H13 were labeled with $Na_2{}^{35}SO_4$ for 10 min in the presence of cycloheximide. Total cell proteins were fractionated by PAGE and autoradiographed. Under the conditions used in Figure 8, two labeled PSI reaction center polypeptides are seen as 2a (*psaB* product) and 2b (*psaA* product) in the wild type lanes (Girard-Bascou *et al.*, 1987). It can be seen in Figure 8 that the synthesis of the reaction center polypeptides is not markedly affected in the transformants relative to wild type. Synthesis of 2b is completely blocked in H13 which is unable to express *psaA*. It is apparent that 2a and 2b in the

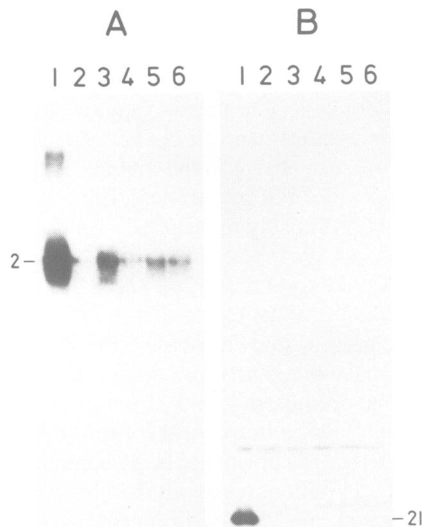

Fig. 7. Western analysis of wild type, H13 and four transformants. Total thylakoid membrane proteins were separated according to Fling and Gregerson (1986) as in Figure 6, electroblotted onto nitrocellulose membrane and reacted with antisera against (**A**) CPI containing the reaction center subunits 2a and 2b and (**B**) P21 corresponding to the *psaF* product. The antibody–antigen complex was visualized with [^{125}I]protein A. 1, wild type; 2, H13; 3, 1B2; 4, 2B2; 5, 1D2; 6, 4B2.

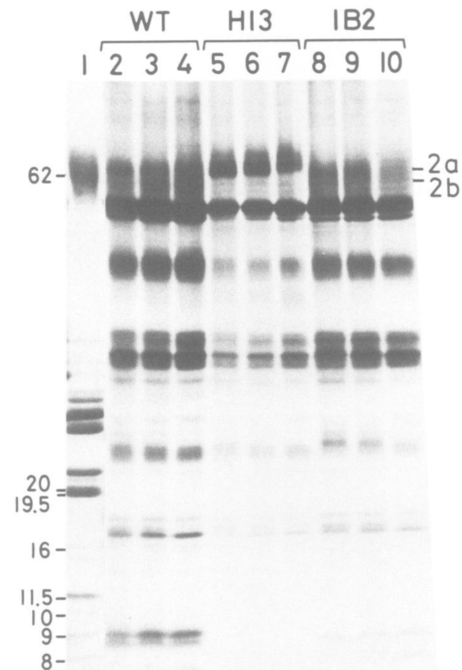

Fig. 8. Pulse–chase labeling of cell proteins. Lane 1, Coomassie blue staining of polypeptides of the purified PSI preparation. Autoradiographs are shown in lanes 2–10. Cells were labeled for 10 min with $Na_2{}^{35}SO_4$ in the presence of 10 μg/ml cycloheximide (lanes 2, 5 and 8) and were then chased for 10 min (lanes 3, 6 and 9) and 60 min (lanes 4, 7 and 10) in the presence of 10 mM Na_2SO_4. Total cell proteins were separated by SDS–PAGE according to Laemmli (1970). The nomenclature of the two reaction center subunits is according to Girard-Bascou *et al.* (1987). The estimated sizes in kd of PSI polypeptides are indicated to the left.

transformant 1B2 are unstable and degraded rapidly during the chase like 2a in H13, while these polypeptides are stable in the wild type. Since the 9 kd protein is strongly labeled with ^{35}S and is synthesized in the presence of cycloheximide, it is likely that it corresponds to the *psaC* product (the *psaC* product should contain 12 sulfur-containing residues besides the initiating methione; see Figure 8). As expected, the corresponding polypeptide is not labeled in the transformant. The absence of label in this polypeptide in H13 is surprising since the primary lesion in this mutant is at the level of *psaA* expression. Complete absence of the *psaA* product could possibly lead to increased turnover of the *psaC* product. It can also be seen in Figure 8 that the incorporation of label in H13 is significantly less than in the wild type and in 1B2.

Discussion

Disruption of the psaC gene in the chloroplast of C.reinhardtii

The recent construction of a chimeric gene expressing spectinomycin resistance in the chloroplast compartment (M.Goldschmidt-Clermont, unpublished results) has allowed us to perform the first directed chloroplast gene disruption on *psaC*. This chimeric gene, consisting of the bacterial gene encoding aminoglycoside 3′ adenyl transferase fused with a 0.65 kb promoter region of *atpA* and of a 0.45 kb downstream region of the *rbcL* gene from *C.reinhardtii*, was inserted within the *psaC* gene. In principle, the presence of chloroplast sequences of *C.reinhardtii* in the selective marker cassette could allow the transferred DNA to integrate into the chloroplast genome of the recipient cells at the corresponding sites by homologous recombination. Events of this sort could be responsible for the additional integrations of the transforming DNA observed as minor bands in the chloroplast DNA molecules of some of the transformants.

One potential problem in a chloroplast disruption experiment is in obtaining a homoplasmic set of modified chloroplast genomes in the transformants. In our experiment, the transformed cells were maintained under selective pressure for many generations and then screened for a PSI deficient phenotype by fluorescence transients, since disruption of *psaC* was expected to abolish PSI function. In this way it was possible to replace all the wild type *psaC* copies with the disrupted gene.

The method for disrupting the *psaC* gene described in the present study is applicable to any other chloroplast gene involved in photosynthesis.

The psaC product is important for stable PSI assembly

At least five chloroplast and six nuclear encoded subunits have been assigned to PSI (Golbeck and Bryant, 1991). Both biochemical and genetic evidence has shown that the two largest subunits encoded by the chloroplast *psaA* and *psaB* genes are part of the reaction center and that they play a crucial role in the stable assembly of the complex (Girard-Bascou *et al.*, 1987). Amongst the numerous nuclear and chloroplast mutants deficient in PSI activity, close to one-quarter are affected specifically in the maturation of the *psaA* mRNA which originates from three separate exons

that are widely separated on the chloroplast genome of *C.reinhardtii* (Kück *et al.*, 1987; Choquet *et al.*, 1988; Goldschmidt-Clermont *et al.*, 1990). Several other mutations specifically affect the synthesis of the *psaA* and *psaB* products at a post-transcriptional level. Four chloroplast loci involved in PSI assembly have been found (Girard-Bascou, 1987). Two of them have been mapped on the chloroplast genome, *psaB* (Girard-Bascou *et al.*, 1987) and *tscA* (Goldschmidt-Clermont *et al.*, 1990; Roitgrund and Mets, 1990). It is possible that one of the remaining loci maps at *psaC*. The remaining PSI mutants of *C.reinhardtii* have not yet been characterized. They may lack one of the constituent PSI polypeptides. Alternatively they may be deficient in a factor involved specifically in the transcription, translation, transport or assembly of the PSI polypeptides. Although these mutations map at different loci they produce the same phenotype: loss of PSI activity and loss of a specific set of PSI polypeptides.

It has been established that the *psaC* product binds the iron sulfur clusters F_A and F_B (Golbeck *et al.*, 1988; Oh-oka *et al.*, 1988; Wynn and Malkin, 1988), yet little is known about its role in the assembly of the PSI complex. In the present study the directed chloroplast transformation allowed us to generate well defined PSI deficient strains whose *psaC* gene is inactivated. Characterization of these transformants has shown that they lack PSI activity and that they no longer accumulate a set of characteristic polypeptides that is missing in other PSI mutants. Pulse labeling of chloroplast encoded proteins of the transformants reveals that the two PSI reaction center subunits are still synthesized at near wild type levels but that they turn over more rapidly than in the wild type, indicating that the *psaC* product is required for the stable assembly of the PSI complex. It has, however, been possible to remove the *psaC* encoded protein from the PSI complex of *Synechococcus* by treatment with chaotropic agents and to reinsert the protein into the PSI core, thereby reconstituting electron transport from P700 to the terminal F_A/F_B acceptors (Golbeck *et al.*, 1988). This apparent contradiction may simply reflect the very different environments of PSI *in vivo* and *in vitro*. It is unlikely that it results from differences in the structure of the PSI complex in *Synechococcus* and *C.reinhardtii* since the PSI core proteins have been highly conserved in these two organisms.

The insertional inactivation of the *psaD* or *psaE* gene has been performed using a transformable cyanobacterium, *Synechocystis sp.* PCC 6803 (Chitnis *et al.*, 1989a,b). The *psaE* gene encodes a peripheral protein located on the stromal side of the thylakoid membranes which corresponds to subunit IV in higher plants or P30 in *C.reinhardtii*. The mutant deficient in this protein showed minor differences in growth under photoautotrophic conditions and in PSI activity compared with the wild type (Chitnis *et al.*, 1989a). The *psaD* gene encodes the ferredoxin docking protein and corresponds to subunit II or P20 which is also located on the stromal side of the thylakoid membranes. The mutant lacking this protein was unable to growth photoautotrophically but had altered PSI activity (Chitnis *et al.*, 1989b). It is likely that this mutant may lack specifically the electron transport activity to ferredoxin. It is noteworthy that although the *psaC*, *psaD* and *psaE* products are peripheral proteins located on the stromal side of the thylakoid membrane, only the first is apparently required for the stable assembly of the PSI complex. These results suggest that the iron sulfur protein is more closely associated with the PSI reaction center protein and is probably surrounded by the *psaD* and *psaE* products to produce a docking site for ferredoxin which accepts electrons from the iron sulfur centers.

Materials and methods

Strains and growth conditions

C.reinhardtii wild type strain 137c and the chloroplast PSI mutant H13 were used in the present work. Growth media, Tris-acetate-phosphate (TAP) and high salt minimal (HSM) were prepared as described (Gorman and Levine, 1965). The transformants were grown in TAP containing 25 μg/ml spectinomycin.

Chloroplast transformation in C.reinhardtii

Chloroplast transformation in *C.reinhardtii* wild type cells was carried out as described by Boynton *et al.* (1988) with a particle gun designed and constructed by Zumbrunn *et al.* (1989). Wild type cells were grown at 25°C on TAP plates under a light intensity of 3000 lux. The cells on the plates were bombarded with tungsten microprojectiles coated with the appropriate DNA. The bombarded cells were incubated at 25°C for a few hours under dim light (300 lux) and then transferred to TAP plates containing spectinomycin (100 μg/ml). After growth for 2 weeks, green colonies appeared and were restreaked on a fresh plate containing spectinomycin. The fluorescence transients of the dark adapted transformed cells were measured, and transformants lacking PSI activity were recloned three times on spectinomycin plates and characterized.

Photosystem I activity measurements

PSI activity was measured by light induced oxygen uptake. The reaction mixture contained 30 μg chlorophyll/ml, 5 mM NaCl, 5 mM $MgCl_2$, 50 mM HEPES–KOH, pH 7.5, 0.2 mM methylviologen, 0.1 mM sodium ascorbate, 0.2 mM 2,6-dichlorophenol indophenol, 3 mM KCN, 2 mM NH_4Cl and 10 μM DCMU. Oxygen uptake activity was measured with a Clark type electrode at 25°C. Fluorescence transients were performed as described (Bennoun and Delepelaire, 1982).

Isolation of total DNA and RNA

Total cellular DNA and RNA were prepared from 50–200 ml TAP cultures shaken continuously at 25°C under dim light (300 lux). Spectinomycin (25 μg/ml) was added to the cultures of transformants. Total DNA and RNA were isolated as described (Weeks *et al.*, 1986; Rochaix *et al.*, 1988).

DNA sequencing

Nucleotide sequences were determined by the chain termination method (Sanger *et al.*, 1977) and by the chemical cleavage method of Maxam and Gilbert (1980).

Isolation of the thylakoid membranes and of the PSI reaction center

The thylakoid membranes were isolated from the wild type, H13 and the transformants according to the method of Chua and Bennoun (1975).

The PSI complex was isolated from wild type thylakoid membranes. The thylakoid membranes were treated with 2 M NaBr in 300 mM sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 50 mM Tris–HCl, pH 7.5 (STMN), on ice for 30 min and subsequently diluted with the same volume of STMN and centrifuged for 30 min at 48 000 *g*. The pellet was washed once with distilled water and then resuspended in distilled water containing 1 mM PMSF (0.8 mg chl/ml). The thylakoid membranes were solubilized with 0.83% *n*-dodecyl-β-D-maltoside on ice for 20 min and then centrifuged to remove undissolved membranes. The supernatant was loaded onto a sucrose density gradient (0.1 – 1 M) containing 5 mM tricine/NaOH, pH 7.5, and 0.05% *n*-dodecyl-β-D-maltoside. After centrifugation for 16 h at 240 000 *g*, the lowest green band containing PSI was isolated. Concentration of chlorophylls was estimated according to the method of Arnon (1949). SDS–PAGE was carried out according to the methods of Fling and Gregerson (1986) and Schägger and Jagow (1987).

Western analysis

CPI antibody was a gift from T.Nechushtai. P21 antibody was obtained after immunizing rabbits with purified P21 protein using poly(A)poly(U) as adjuvant (Hovanessian *et al.*, 1988). After electrophoretic fractionation, the proteins were electroblotted onto nitrocellulose membranes, reacted with

antisera and subsequently with [^{125}I]protein A to reveal the antibody–antigen complexes.

Pulse–chase labeling experiments

Cells were grown under dim light (300 lux) to exponential phase ($5 \times 10^5 - 1 \times 10^6$ cells/ml) in TAP medium containing $MgCl_2$ in place of $MgSO_4$ and for transformant cultures 25 μg/ml spectinomycin was added. Cells were washed and then resuspended at 1.5×10^7 cells/ml in TAP lacking sulfate. Cultures were first continuously shaken for 2 h and then cycloheximide was added (10 μg/ml) for 5 min. Pulse labeling of cells was performed with carrier-free $Na_2{}^{35}SO_4$ (0.5 mCi/ml) for 10 min and the chase was initiated by addition of 10 mM Na_2SO_4. Aliquots of cultures were immediately chilled, centrifuged and the cells were frozen at −20°C. Labeled proteins were solubilized with 2% SDS and 0.1 M dithiothreitol and boiled for 1 min. Proteins were fractionated by SDS–PAGE according to Laemmli (1970).

S1 mapping and primer extension

S1 mapping was carried out according to Sambrook *et al.* (1989) and primer extension was according to Ausubel *et al.* (1990). A 3.8 kb *Pst*I–*Nco*I fragment was 3′ end-labeled at the *Nco*I site and a 0.8 kb *Nco*I–*Sal*I and a 1.2 kb *Sal*I–*Eco*RI fragments were 5′ end-labeled at the *Nco*I and *Sal*I sites respectively and used as probes for S1 mapping (see Figure 1). Two different oligonucleotides complementary to parts of *psaC*, 5′-AACCAATACTAGTATCGTA and 5′-CAACCATCCCATGGAACCATTT, were used for primer extension.

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