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Transformation of chloroplasts with the *psa*B gene encoding a polypeptide of the photosystem I reaction center

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A chloroplast photosystem I reaction center mutation, *ac-u-g-2.3*, of *Chlamydomonas reinhardtii* has been complemented with a wild type *psa*B gene to restore photosynthetic competence. The mutation was mapped in the *psa*B coding sequence by chloroplast transformation using subcloned restriction fragments of *psa*B. The mutation was found to be a single base pair deletion resulting in a reading frame shift and premature termination of the polypeptide. Transformants were verified by insertion of a site-directed mutation which created a new restriction enzyme site. These transformations demontrate the feasibility of insertion of site-directed mutations into the *psa*B gene in order to elucidate amino acid residues involved in photosystem I assembly and function.

Transformation; Chloroplast; Complementation; Photosystem I; *Chlamydomonas reinhardtii*

1. INTRODUCTION

Photosystem I of plants and algae utilizes light energy to transfer electrons from plastocyanin to ferredoxin. The primary electron donor, P700, as well as the electron transfer components A_o, A_1, and iron sulfur center F_x are all bound to two related polypeptides of 82–84 kDa, called IA and IB. The genes encoding these polypeptides are designated *psa*A and *psa*B and have been sequenced from a variety of organisms (for a review see [1]). Despite this detailed information on the primary sequence of the photosystem I reaction center polypeptides from diverse organisms, the nature and involvement of specific amino acid residues in co-factor binding is mostly unknown. Our understanding of photosystem I would be greatly improved by developing a system in which site-directed mutagenesis could be used to test predictions of structure-function relationships in the photosystem I reaction center.

Chlamydomonas reinhardtii provides a system particularly amenable to study photosystem I function. The biolistics technique has been used to transform its chloroplast allowing introduction of genes into the plastid genome [2]. In addition, since *C. reinhardtii* can grow heterotrophically using acetate as a carbon source, it has been possible to isolate many photosystem I mutants [3,4]. In this paper we report the molecular characterization of one such photosystem I mutant, *ac-u-g-2.3* [5]. Chloroplast transformation was used to map the genetic locus of the mutation within the *psa*B coding sequence; the mutation was found to arise from a single base pair deletion in *psa*B resulting in a reading frame shift and premature termination of polypeptide elongation. Our results indicate that mutated *psa*B sequences can be stably introduced into the chloroplast genome of *C. reinhardtii* demonstrating the feasibility of site-directed mutagenesis of *psa*B in this green alga.

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2. MATERIALS AND METHODS

2.1. *Organism and culture conditions*

Chlamydomonas reinhardtii strain cc2341 (*ac-u-g-2.3*) was obtained from the *Chlamydomonas* culture collection at Duke University. Cultures were maintained at approximately 100 μE $m^{-2} \cdot s^{-1}$ on the acetate-supplemented medium of Sueoka [6] prior to transformation. Following transformation cells were subjected to a 3-fold higher light intensity on the same medium lacking a carbon source.

2.2. *Chloroplast transformation*

C. reinhardtii was transformed by tungsten particle bombardment using the DuPont PDS1000 Particle Delivery System. Methods were essentially as described previously [2,7] except that routinely $1–5 \times 10^7$ cells were bombarded directly on selective plates (lacking acetate). Photosynthetic colonies were twice streaked on selective plates prior to DNA isolation.

2.3. *Site directed mutagenesis and DNA sequencing*

Site-directed mutagenesis utilized the Amersham Oligonucleotide-directed mutagenesis kit according to the manufacturer's protocol. Standard polymerase chain reaction (PCR) amplifications were designed according to established procedures [8]. PCR products were eluted from low-melting agarose gels using an elutip column (Schleicher and Schuell, Keene, NH) and the double-stranded DNA sequenced [9].

2.4. *DNA isolation and Southern blot analysis*

Rapid mini-preparations of total *C. reinhardtii* DNA were prepared

from 10–50 ml cultures as described previously [10]. Southern blot hybridizations employed standard methodology [8].

3. RESULTS

The *ac-u-g-2.3* mutation was generated by fluorodeoxyuridine treatment of wild type *C. reinhardtii* and subsequent sodium arsenate enrichment for non-photosynthetic cells [5]. Since chlorophyll protein complex 1 (CP1) was missing from polyacrylamide gels of its thylakoid polypeptides, the *ac-u-g* locus was identified as a photosystem I mutation. Subsequent recombination tests between *ac-u-g-2.3* and other chloroplast DNA markers demonstrated that this mutation was allelic to a known *psa*B mutation, FUD26 [4]. These mutations are tightly linked to the 10-6C mutation localized within the *rbc*L gene encoding the large subunit of ribulose-bisphosphate carboxylase. Both the *psa*B and *rbc*L genes are located on the same 5.8 kbp *Eco*RI fragment, *Eco*14 [11] (Fig. 1). Employing the *ac-u-g-2.3* mutant strain, we first sought to demonstrate that this mutant could be rescued by transformation with the wild type *psa*B gene. *Eco*RI fragment 14 from wild type *C. reinhardtii* chloroplast DNA (Fig. 1) was used for particle bombardment of strain cc2341. Photosynthetic colonies appeared at a frequency in the range of 1×10^{-5}–10^{-6} far above a spontaneous reversion frequency of approximately 1×10^{-8} (Table I).

In order to localize the mutation within the *psa*B sequence, *Eco*14 was subcloned, dividing the *psa*B coding sequence into three regions, 14.1, 14.2 and 14.3 (Fig. 1). These plasmids were again bombarded into cc2341. Only bombardment with plasmid 14.3 yielded photosynthetic colonies indicating that the *ac-u-g-2.3* mutation was limited to either the 3′ terminal coding region or the 3′ noncoding sequences of *psa*B (Table I).

A 678 bp fragment containing the 3′ coding sequence of *psa*B was amplified by PCR from a miniprep of total

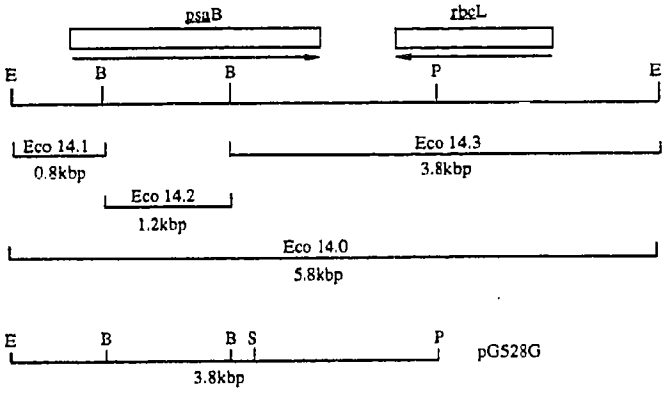

Fig. 1. Restriction endonuclease map of the 5.8 kbp *Eco*RI fragment 14 from *C. reinhardtii* chloroplast DNA. The position and size of three subclones of fragment *Eco*14, called 14.1, 14.2 and 14.3 used to transform *C. reinhardtii* are indicated. pG528G is the 3.8 kbp subclone of *Eco*14 used for site-directed mutagenesis and transformation of *C. reinhardtii*. E, *Eco*RI; B, *Bam*HI; S, *Stu*I; P, *Pst*I.

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513
Thr Ile Gly Pro Gly Asp Phe Leu Val His His Ala Ile Ala Leu
ACT ATT GGC CCT GGT GAC TTC CTT GTT CAC CAC GCT ATT GCT TTA
ACT ATT GGC CCT GGT GAC TTC CTT GTT CAC CAC GCT ATT GCT TTA
Thr Ile Gly Pro Gly Asp Phe Leu Val His His Ala Ile Ala Leu

528
Gly Leu His Thr Thr Thr Leu Ile Leu Val Lys Gly Ala Leu Asp
GGT CTT CAC ACT ACA ACA TTA ATC CTT GTT AAA GGT GCT CTT GAT
GGT CTT CAC ACT ACA ACA TTA ATC CTT GTT AAA GGT GCT CTT GAT
Gly Leu His Thr Thr Thr Leu Ile Leu Val Lys Gly Ala Leu Asp

543
Ala Arg Gly Ser Lys Leu Met Pro Asp Lys Lys Asp Phe Gly Tyr
GCT CGT GGT TCT AAA CTA ATG CCA GAT AAA AAA GAC TTT GGT TAC
GCT CGT GGT TCT AAA CTA ATG CCA GAT AAA AAG ACT TTG GTT ACA
Ala Arg Gly Ser Lys Leu Met Pro Asp Lys Lys Thr Leu Val Thr

558
Ser Phe Pro Cys Asp Gly Pro Gly Arg Gly Gly Thr Cys Asp Ile
AGC TTC CCA TGT GAC GGT CCT GGT CGT GGC GGT ACT TGT GAC ATT
GCT TCC CAT GTG ACG GTC CTG GTC GTG GCG CGG GTA GTG ACA TTT
Ala Ser His Val Thr Val Leu Val Val Ala Val Leu Val Thr Phe

573
Ser Ala Tyr Asp Ala Phe Tyr Leu Ala
TCA GCT TAC GAT GCT TTC TAC TTA GCT
CAG CTT ACG ATG CTT TCT ACT TAG CTG
Gln Leu Thr Met Leu Ser Thr  *
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Fig. 2. Partial nucleotide and deduced amino acid sequence of the *psa*B gene from wild type (top) and strain cc2341 (bottom) carrying the *ac-u-g-2.3* mutation. The deletion with resulting frameshift at codon number 553 is in bold type. The domain binding the iron-sulfur center, F_x, is underlined.

cellular DNA isolated from strain cc2341. This fragment was gel-purified and both strands of the eluted DNA sequenced. Although the wild type *psa*B gene had been sequenced previously [12], the equivalent region of wild *psa*B, used for our transformation experiments, was also sequenced for comparison to the mutant. A deletion of a single AT base pair in a series of 6 AT base pairs was the only difference between mutant and wild type genes (Fig. 2). Based upon this sequence information, the deletion induces a frameshift and premature termination of the polypeptide at codon position 580. The truncated polypeptide is 157 amino acids shorter than wild type. The same fragment from a randomly chosen transformant was also amplified and sequenced. As expected, the AT bp, which was deleted in the mutant, was restored making the transformant *psa*B gene identical to wild type.

There are no identifiable restriction fragment polymorphisms available in *psa*B when both donor DNA

Table I

Summary of transformations at the *psa*B locus

Transforming DNA	Photosynthetic colonies*
*Eco*14.0	105,160
*Eco*14.1	0,0
*Eco*14.2	0,0
*Eco*14.3	16,24
pG528G	96,136
M13RF(G528G)	45,14
M13ss(G528G)	24,35

*Numbers separated by a comma represent separate experiments where 5×10^7 cells were transformed. Transforming DNAs are depicted in Fig. 1.

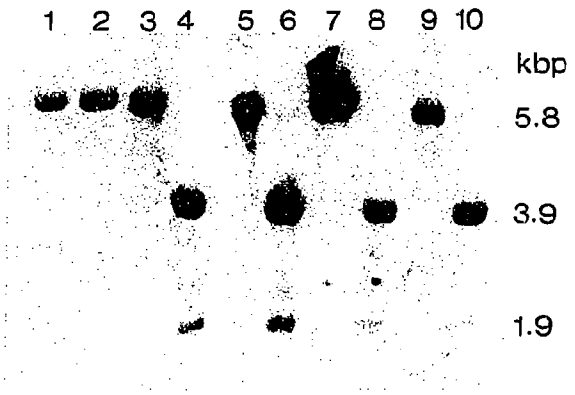

Fig. 3. Southern blot hybridization of *C. reinhardtii* DNA digested with restriction enzymes, separated on a 0.8% agarose gel and probed with pG528G. Lanes 1 and 2 contain DNA from untransformed strain cc2341. Lanes 3 and 4, 5 and 6, 7 and 8, and 9 and 10 contain DNA from four randomly chosen transformants, respectively. DNA in odd numbered lanes was digested with *Eco*RI. DNA in even numbered lanes was digested with both *Eco*RI and *Stu*I.

and recipient chloroplast chromosome originate from *C. reinhardtii*. Therefore, true transformants cannot be distinguished from rare, but nevertheless plausible, revertants. In order to identify the authentic transformants, a silent, site-directed mutation, creating a single *Stu*I restriction endonuclease site, was introduced into *psa*B at glycine codon 528. The mutation was generated in a 3.8 kbp *Eco*RI–*Pst*I fragment, subcloned from M13mp19 into plasmid vector pUC19 (called pG528G) (Fig. 1) and bombarded into cc2341. The frequency of appearance of photosynthetic colonies was nearly as high using pG528G as with E14 (Table I). Both the single-stranded and the RF forms of M13 carrying the G528G mutation within the 3.8 kbp fragment also produced photosynthetic colonies following particle bombardment, although at a reduced frequency compared to the plasmid construction (Table I).

Photosynthetic colonies were randomly chosen and minipreps of total DNA digested with restriction enzymes, run on agarose gels, and blotted for Southern hybridizations using pG528G as a probe. Digestion of DNA from untransformed cc2341 with *Eco*RI or double digestion with *Eco*RI and *Stu*I yielded the single 5.8 kbp fragment 14, indicating that, as expected, there is normally no *Stu*I site within *psa*B (Fig. 3, lanes 1 and 2). Hybridization to *Eco*RI digests of four transformants yielded the same 5.8 kbp fragment 14 (Fig. 3, lanes 3,5,7,9). However, two fragments of 3.9 and 1.9 kbp were generated in each transformant lane following the *Eco*RI–*Stu*I double digest (lanes 4,6,8,10). These fragments are of the predicted size if the wild type *psa*B carrying the engineered *Stu*I site has recombined with chloroplast DNA replacing all or part of the mutant *psa*B of cc2341. No vector sequences of either plasmid or phage origin were detected in any transformants.

Photosynthetic growth by the transformants clearly provides an effective selection for segregation of the wild type *psa*B DNA throughout all copies of the chloroplast genome. Most of the transformant DNAs analyzed digested completely with *Stu*I (Fig. 3, lanes 4,8,10) indicating complete segregation of the transformed DNA. Occasionally (Fig. 3, lane 6) faint partial *Stu*I digests were detected in Southern blots; however, these instances appear to be due to partial enzyme digests of completely segregated chloroplast DNA preparations since the PCR-amplified *psa*B gene from these same preparations digested completely with *Stu*I (not shown).

4. DISCUSSION

This report documents the first instance in which an organism has been transformed using a photosystem I reaction center gene demonstrating the utility of the system for genetic engineering of this complex. The wild type *C. reinhardtii psa*B gene and a subcloned fragment encompassing the 3′ 800 bp of *psa*B both complement the *ac-u-g-2.3* mutation at high frequency. A site-directed mutation creating a unique *Stu*I restriction endonuclease site within *psa*B has been used to identify transformants. Results of Southern blot hybridizations to transformant DNAs (Fig. 3) indicate that the *Stu*I site has been introduced into the chloroplast chromosome by homologous recombination similar to observations reported when other chloroplast DNA fragments have been used to transform *C. reinhardtii* [2,7,13,14].

Sequence analysis of the *ac-u-g-2.3* mutation indicates that a single AT bp deletion results in a reading frameshift and premature termination of polypeptide chain elongation. A similar delection of four AT bp causes premature termination of this polypeptide in a previously characterized *C. reinhardtii* mutant FUD26 [15].

The engineered *Stu*I site has been introduced into the *psa*B gene 25 codons upstream of the *ac-u-g-2.3* mutation and 32 codons from the amino acid domain proposed to bind the iron sulfur center, F_x (Fig. 2). The intervening amino acid residues are highly conserved in oxygenic photosynthetic organisms [1] and possibly contain residues involved in binding the primary electron donor, P700 and electron transport components A_0 and A_1. In addition, Webber and Malkin [16] have proposed that a leucine zipper motif in this region facilitates interaction of the reaction center heterodimer. Therefore, we will be able to utilize the *Stu*I site as a transformation marker and the *ac-u-g-2.3* strain as a convenient recipient of further site-directed mutations designed to elucidate those residues coded in *psa*B which are directly involved in photosystem I assembly and function.

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