Plant Cell Physiol. 37(2): 161-168 (1996)

Genetic Engineering of the Processing Site of D1 Precursor Protein of Photosystem II Reaction Center in *Chlamydomonas reinhardtii*

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The D1 protein (D1) of photosystem II (PSII) reaction center is synthesized as a precursor (pD1) and then processed at its carboxyl terminus to establish the function of water cleavage. The amino acid sequence of the carboxyl terminal extension excised by this process is poorly conserved except for a residue after the cleavage site at position of 345. We have constructed a vector for site-directed mutagenesis of the chloroplast *psbA* gene encoding D1 of the green alga, *Chlamydomonas reinhardtii*. The vector enables one to transform the chloroplasts of a *psbA* deletion mutant (Fud7) and directly select transformants for resistance to spectinomycin. Using this transforming vector, we have substituted Ser345 to Gly, Cys, Val and Phe in order to investigate effects of the amino acid side chain at this position on the processing rate. All of the resulting transformants exhibited the PSII activity as wild type and grew normally under photoautotrophic conditions even under strong light where rapid turnover of D1 protein is expected to occur. Western blotting analysis demonstrated that mature D1 accumulates in these transformants at wild type level. Pulse and chase labeling of chloroplast-encoded proteins using [^{35}S]sulfate revealed that the processing of D1 precursor protein occurs in all four transformants as efficiently as in wild type, at least under the experimental conditions examined. The results suggest that either the amino acid side chain at position of 345 (+1 position) is not crucial to the enzymatic cleavage of pD1 in vivo or the apparent rate of processing in vivo is not limited by the enzymatic cleavage.

Key words: C-terminal processing — *Chlamydomonas reinhardtii* — Chloroplast transformation — D1 protein — Photosystem II — *psbA*.

The isolated photosystem II (PSII) reaction center consists of two structurally homologous proteins called D1 and D2, cytochrome b_{559} and *psbI* gene product (Nanba and Satoh 1987). The D1 was discovered as one of the most rapidly synthesized proteins in the chloroplast (Ellis 1981) and also known as a herbicide binding protein (Pfister et al. 1981). This protein is translated as a precursor (pD1) (Minami and Watanabe 1985, Reisfeld et al. 1982), integrated into the thylakoid membranes and then processed at its carboxyl terminus (Marder et al. 1984, Takahashi et al. 1989, 1990). Although the D1 is synthesized abundantly under illumination, it is accumulated stoichiometrically in the PSII reaction center complex, i.e., equimolar ratio to D2, because of its rapid turnover (Mattoo et al. 1989).

Determination of carboxyl terminal residue of D1 revealed that the precursor protein is processed at the carboxyl side of Ala at position of 344 (Takahashi et al. 1989, 1990), resulting in the removal of carboxyl terminal extension with 8-16 amino acids. The processing of pD1 is absolutely required for the assembly of manganese cluster involved in the oxygen evolution in PSII (Taylor et al. 1988). However, physiological significance of the presence of carboxyl terminal extension in D1 has not been established at present. Transformants from a green alga, *Chlamydomonas reinhardtii* and a cyanobacterium, *Synechocystis* sp. PCC 6803, where stop codon is introduced at position of 345 (+1 position), were found to grow normally under photoautotrophic conditions (Lers et al. 1992, Nixon et al. 1992, Schrader and Johanningmeier 1992). In addition, the nucleotide sequence of *Euglena gracilis psbA* gene predicts that there is no carboxyl terminal extension in the D1 of this organism (Karabin et al. 1984, Keller and Stutz 1984). These results suggest that the carboxyl terminal extension of pD1 is dispensable for assembly of functional PSII complex. However, removal of the extension is evidently indispensable, if it is present, as shown by LF-1 mutant of *Scenedesmus obliquus* (Taylor et al. 1988) and site-directed mutants of *Synechocystis* sp. PCC 6803 (Nixon et al. 1992). On the other hand, in an in vitro analysis using partially purified processing protease and synthetic oligopeptides, amino acid substitutions at the cleavage site appreciably influenced the rate of carboxyl terminal excision (Taguchi et al. 1995).

Comparison of amino acid sequences of D1 deduced from *psbA* gene of different organisms examined so far reveals that the carboxyl terminal sequence of mature protein is highly conserved. On the contrary, the sequence, as well as the chain length, of carboxyl extension is poorly conserved, except for a residue at +1 position of pD1; Ala in most cases and Ser in *C. reinhardtii* and some cyanobacteria (Svensson et al. 1991).

In the present study, we have mutated Ser345 in pD1 of *C. reinhardtii* to Gly, Cys, Val and Phe to investigate the effect of amino acid substitutions at this position on

the processing of pD1 in vivo. The result demonstrated that these substitutions affected neither photoautotrophic growth of the cells nor the rate of processing reaction of pD1 at least under the conditions examined.

Materials and Methods

Strains and growth media—C. reinhardtii wild-type strain 137c and the chloroplast *psbA* deletion mutant Fud7 were used in the present study. Cells were grown on either Tris-acetate-phosphate (TAP) or high-salt minimal (HSM) medium as descried (Gorman and Levine 1965). The transformants were selected on TAP plates containing 150 μg ml^{-1} spectinomycin and were maintained on TAP plates containing 25 μg ml^{-1} spectinomycin.

DNA and plasmids—Procedures for preparation of recombinant DNA plasmids are described in (Sambrook et al. 1989). For construction of plasmid to transform *psbA* deletion mutant Fud7, 5.0 kb *Eco*RI/*Xho*I fragment containing exon 5 and 3′ flanking region of the *psbA* was cloned in the Bluescript vector (Stratagene) from *Chlamydomonas* chloroplast *Bam*HI fragment Ba12 according to Rochaix (Rochaix 1978). The *aadA* cassette from pUC-atpX-AAD was inserted at *Bam*HI restriction site downstream of the *psbA* gene in the opposite orientation with the *psbA* gene (pEX-50-AAD). Since the *aadA* cassette was excised by digestion with *Eco*RV and *Xba*I, *Bam*HI restriction site was recreated in the 3′ end of the *aadA* cassette. A 6 kb *Eco*RI fragment R14 according to Rochaix containing exons 1–4 of the *psbA* gene was subsequently inserted at *Eco*RI site in the pEX-50-AAD. The resulting plasmid pR12-EX-50-AAD entirely covers a deletion that ranges from a part of the first intron extending to the 3′ flanking region of the *psbA* gene in chloroplasts of Fud7.

A 1.8 kb *Bam*HI-*Xba*I fragment containing 3′ part of the *psbA* exon 5 was cloned in Bluescript (pBX-5) for introduction of point mutations that replace Ser345. Single strand DNA containing this region was produced using helper phage as described in manufacturer's protocol and was used as a template for in vitro oligonucleotide-directed mutagenesis (Sculptor in vitro mutagenesis system, Amersham). Replacement of Ser345 to Gly, Cys, Val and Phe was performed using oligonucleotides, 5′-CTAGACTTAGCTGGTACTAACTCTAGC-3′, 5′-TAGACTTAGCTTGTACTAACTCTAGC-3′, 5′-CTAGACTTAGCTGTAACTAACTCTAG-3′ and 5′-TAGACTTAGCTTTCACTAACTCTAGC-3′, respectively (underline indicates the mutated nucleotides). Since the introns of the *psbA* gene are dispensable (Johanningmeier and Heiss 1993, Minagawa and Crofts 1994), it was confirm by sequencing that no other nucleotides in the exon5 were modified during mutagenesis reaction.

To introduce a DNA fragment with mutations in the pR12-EX-AAD plasmid, the *Bam*HI/*Xba*I wild type fragment in the pEX-50-AAD was replaced by the corresponding fragment with mutations. R14 fragment was added into the resulting plasmid containing mutations at *Eco*RI site as described above.

Chloroplast transformation—Chloroplast transformation was performed with a particle gun (Nippon Zeon Co.) as described (Boynton et al. 1988, Takahashi et al. 1994). Host cells (Fud7) were grown in TAP culture under dim light (1–2 × 10^6 cells ml^{-1}), pelleted by centrifugation and resuspended in a smaller volume of TAP medium (2–4 × 10^7 cells ml^{-1}). The resultant concentrated cells were plated on TAP plates, subsequently grown for 2 days under dim light and were then used for bombardment. After bombardment, cells on the plate were collected and transferred onto the TAP plates containing 150 μg ml^{-1} spectinomycin. The transformants were recloned three to four times until they were homoplasmic.

Isolation of total DNA—Total DNA of the transformants was prepared from 100–150 ml TAP cultures containing 25 μg ml^{-1} spectinomycin under dim light as described (Weeks et al. 1986). The DNA fragment of *psb*A exon 5 was amplified by polymerase chain reaction using oligonucleotides; 5′-GGTTACTTTGGTCGTCTAATCTTCCAATAC-3′ and 5′-TTAGTTGTTTGAGCTAGAGTTAGTTGAAGC-3′.

Western analysis—Total cell proteins were separated by SDS-polyacrylamide gel electrophoresis according to (Laemmli 1970), electroblotted on nitrocellulose filters, reacted with antiserum and visualized by the enhanced chemiluminescence (ECL) method (Amersham).

Pulse-labeling of cells—Cells were grown under dim light to exponential phase (1–3 × 10^6 cells ml^{-1}) in TAP medium containing reduced level of sulfate as described (Takahashi et al. 1991). The cells were washed once and then resuspended in TAP lacking sulfate (1 × 10^7 cells ml^{-1}). Cultures were continuously shaken for 2–3 h and then cycloheximide was added (10 μg ml^{-1}) 5 min prior to labeling. Pulse labeling of cells was carried out with carrier-free [^{35}S]Na_2SO_4 (0.37 MBq ml^{-1}) for 3 min and terminated by addition of 10 mM Na_2SO_4 and chloramphenicol (200 μg ml^{-1}). Total cell proteins were solubilized with 2% SDS and 0.1 M dithiothreitol at 100°C for 1 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis containing 6 M urea and 12.5 or 15% acrylamide in the resolving gel.

Measurements of PSII activities—Light-induced O_2-evolving activity was measured with a Clark type electrode as described (Takahashi et al. 1994). Chlorophyll concentration was determined as described (Arnon 1949). Fluorescence induction transients of cells adapted to the dark for 10 min were measured using Plant Efficiency Analyzer (Hansatech).

Results

Construction of a vector for site-directed mutagenesis of the chloroplast psbA gene—In *Chlamydomonas reinhardtii*, the chloroplast *psbA* gene is present in the inverted repeats and contains four introns as shown in Fig. 1 (Erickson et al. 1984). To engineer the chloroplast *psbA* gene, a 5.0 kb *Eco*RI-*Xho*I DNA fragment containing the *psbA* exon 5 and its 3′ region was cloned in a plasmid Bluescript. The *aadA* cassette that confers an antibiotic resistance in the chloroplast (Goldschmidt-Clermont 1991) was inserted at *Bam*HI restriction site in approximately 1.7 kb downstream of the *psbA* termination codon (Fig. 1). No possible secondary effect to photosynthetic activity caused by insertion of the *aadA* cassette was confirmed by the fact that the transformants can grow on minimal medium (HSM) in the light at wild type level and that fluorescence induction transients were normal (see Fig. 4).

Although this construct can be used to introduce a mutation into the exon 5 using wild type cells as a recipient strain, we subsequently added a 6.0 kb *Eco*RI fragment containing the remainder of the *psbA* gene and its 5′ flanking region. The resulting construct allows one to use a *psbA* deletion mutant, Fud7, as a host for directed transfor-

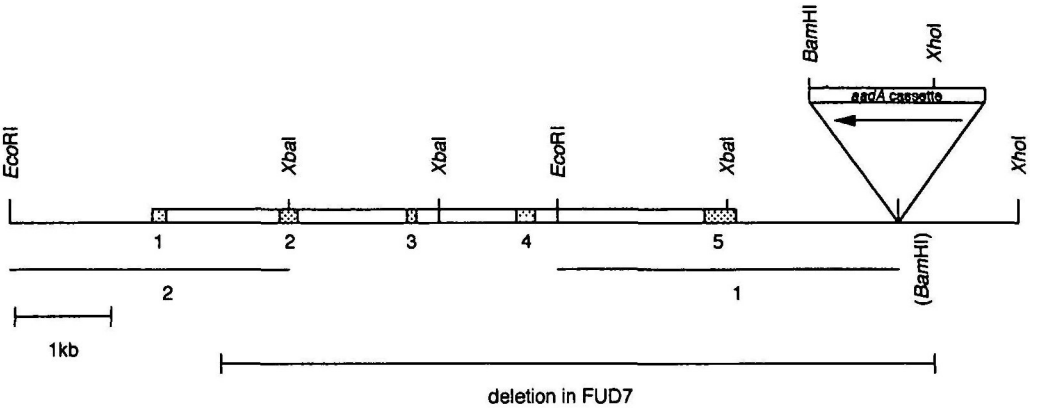

Fig. 1 The vector for site directed mutagenesis of the *psbA* gene. The 11 kb *Eco*RI and *Xho*I fragment with the *aadA* cassette inserted at *Bam*HI site downstream of the *psbA* gene was cloned in plasmid Bluescript. The *Bam*HI site was recreated in the 5′ end of the *aadA* cassette. Bar in the bottom indicates a deletion in Fud7 strain. The 1.8 kb *Bam*HI-*Xba*I fragment was used as template for site-directed mutagenesis which replace Ser at position of 345 to either Cys, Gly, Val or Phe. Bars 1 and 2 represent probes 1 and 2, respectively, which were used for Southern analysis in Fig. 3.

mation (Bennoun et al. 1986).

Determination of the carboxyl terminus residue of D1 indicated that pD1 is processed between Ala and Ser at the positions of 344 and 345, respectively, in *C. reinhardtii* (unpublished result, see (Nixon et al. 1992)). In order to investigate significance of Ser residue at +1 position on pD1 processing, we have substituted this residue by site-directed mutagenesis of the chloroplast *psbA* gene in *C. reinhardtii* using the transforming vector constructed as described above. We have substituted it to Gly, Cys, Val and Phe (Fig. 2). These four mutations were generated by oligonucleotide directed mutagenesis using 1.8 kb *Bam*HI-*Xba*I fragment containing 3′ part of the exon 5 of the *psbA* as a template. The resulting *Bam*HI-*Xba*I DNA fragment with a mutation was replaced with the corresponding wild type fragment in the transforming vector. Using these vectors, we transformed chloroplasts of Fud7 cells by particle gun-mediated transformation and putative transformants were selected for resistance to spectinomycin.

Figs. 3A and B show Southern blotting analysis of wild type, Fud7 and the five different transformants. Total DNA was digested by *Eco*RI, separated by agarose gel electrophoresis and subsequently blotted onto nitrocellulose filter. When hybridized with probe 1 (see also Fig. 1), 6.0 kb signal was observed in wild type and the transformants, while no signal was detected in Fud7 (Fig. 3A). Fud7 gave rise to 3.5 kb signal when hybridized by probe 2 as expected (Fig. 3B). These results, together with the absence of the 3.5 kb signal in the transformants, indicate that the sequence deleted in Fud7 was recovered by homologous recombination.

Since a new *Rsa*I restriction site is generated in the mutants of Ser345Cys and Ser345Gly, we examined the occurrence of this new restriction site in the transformants. We have amplified the DNA sequence of the exon 5 by polymerase chain reaction using total DNA isolated from wild type and the transformants, and the resulting DNA fragments of 300 bp were digested by *Rsa*I (Fig. 3C). The digestion of wild type fragment resulted in two fragments of 180 and 120 bp while the fragment of 120 bp from the Ser345Cys and Ser345Gly transformants was replaced by two bands of 105 and 15 kb as expected. We also determined the DNA sequences of the exon 5 from four transformants using the amplified DNA fragments (data not

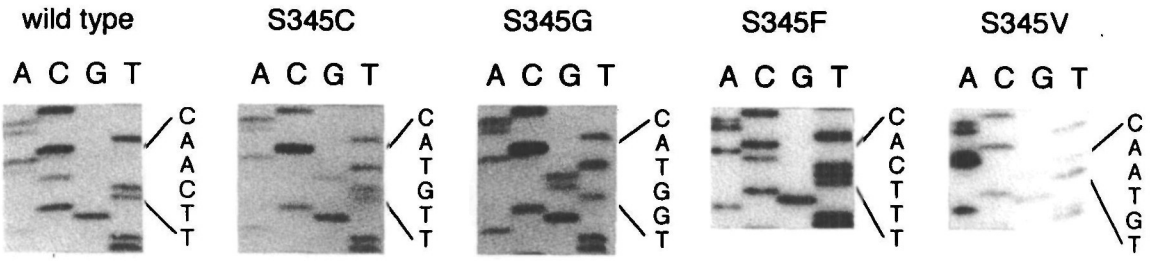

Fig. 2 Mutations of the *psbA* gene. Ser at the position of 345 was replaced by either Gly (S345G), Cys (S345C), Val (S345V) or Phe (S345F). A new restriction site *Rsa*I which recognizes GTAC was created in S345C and S345G transformants.

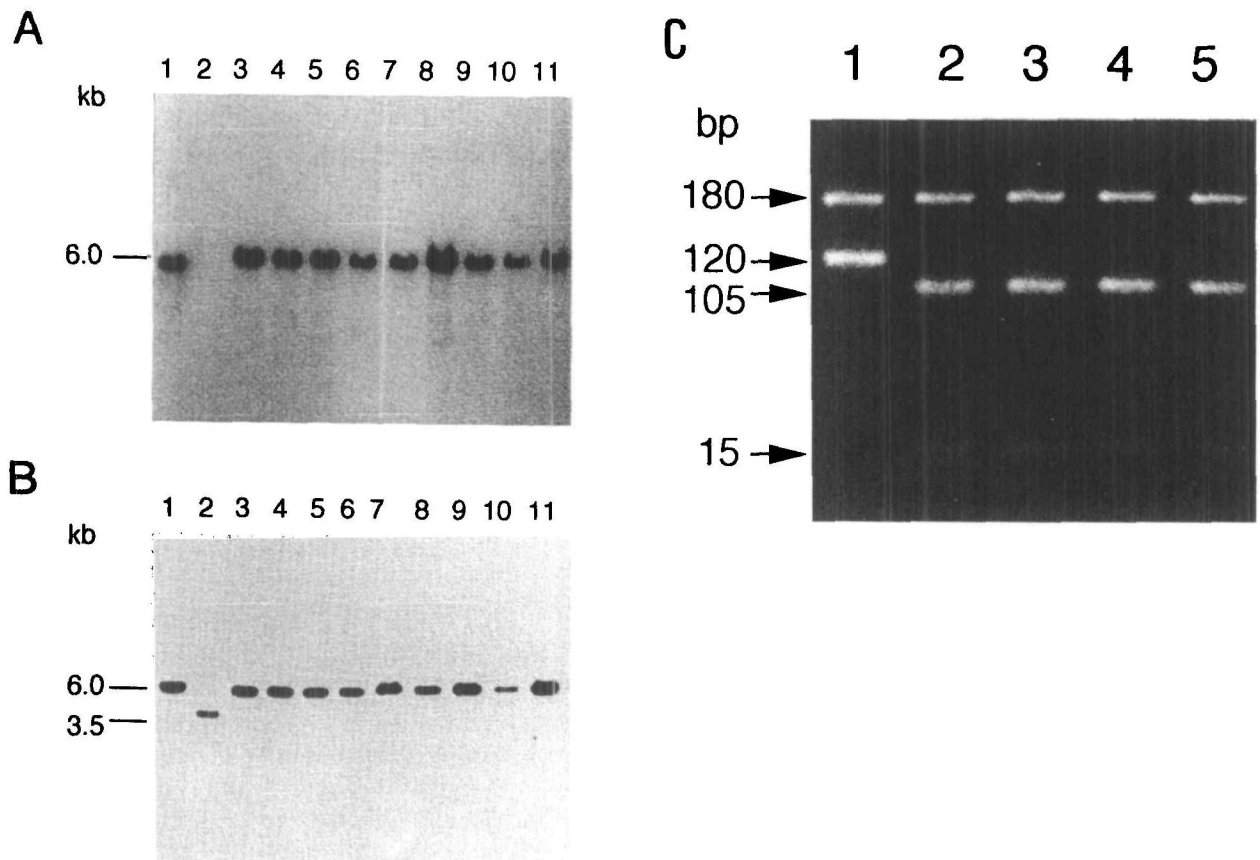

Fig. 3 (A and B) Southern analysis of the total cellular DNA from the transformants. Total DNA from the transformants were digested by *Eco*RI, fractionated by agarose gel electrophoresis and blotted on nitrocellulose filter. 1, wild type; 2, Fud7; 3, the transformant with wild type *psbA* gene and the *aadA* cassette (control); 4 and 5, Ser345Gly; 6 and 7, Ser345Cys; 8 and 9, Ser345Val; 10 and 11, Ser345Phe. The blots were hybridized with a probe (A) 3.7 kb *Bam*HI-*Eco*RI DNA fragment (probe 1 in Fig. 1), and (B) 1.8 kb *Eco*RI-*Xba*I DNA fragment (probe 2 in Fig. 1). (C) Digestion of the exon 5 fragments from Ser345Cys and Ser345Gly transformants. 300 bp DNA fragments of the exon 5 were generated by amplification using polymerase chain reactions with total cellular DNA from Ser345Cys and Ser345Gly mutants as templates. The resulting DNA fragments were digested by *Rsa*I and subsequently separated on 2.5% agarose gel electrophoresis. 1; wild type, 2 and 3; Ser345Cys, 4 and 5; Ser345Gly. Wild type DNA fragment showed two bands of 180 and 120 bp while the transformant DNA fragments showed three bands of 180, 105 and 15 bp.

shown).

Substitutions of Ser345 affect no photosynthetic phenotype—To investigate steady-state accumulation of D1, total cell proteins of the transformant cells grown to mid-log phase in TAP liquid culture were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose filter and probed with an antibody raised against D1 of wild type strain. Fig. 4 shows that, while the D1 is totally absent in Fud7 cells as expected, all transformants accumulated D1 at wild type level. It is also noted that migration of the band hybridized with anti-D1 antibody in each transformant is identical to that of mature D1 in wild type, suggesting that pD1 in these transformants is processed correctly.

Fluorescence induction transients of the dark adapted cells of the transformants were compared to examine effects of these amino acid substitutions on their photosynthetic activity (Fig. 5). All four transformants exhibited wild type fluorescence transients and wild type ratio of variable fluorescece to maxium fluorescence (F_v/F_{max}) of 0.6, indicating that overall photosynthetic electron transfer activity around PSII is not altered by these mutations. The four transformants also had oxygen evolving activity of 60–70 μmol O_2 mgchl^{-1} h^{-1}, which is comparable to that of wild type (70 μmol O_2 mgchl^{-1} h^{-1}).

To test if cells of the transformants can grow photoautotrophically, they were spotted on minimal plate in the light (Fig. 6). These four transformant cells appeared to grow like wild type even under strong light of 50,000 lux where the D1 is expected to turn over rapidly (Marder et al. 1984).

In order to investigate rates of cleavage of pD1 in the transformant cells quantitatively and more directly, the chloroplast encoded proteins were pulse labeled for 3 min

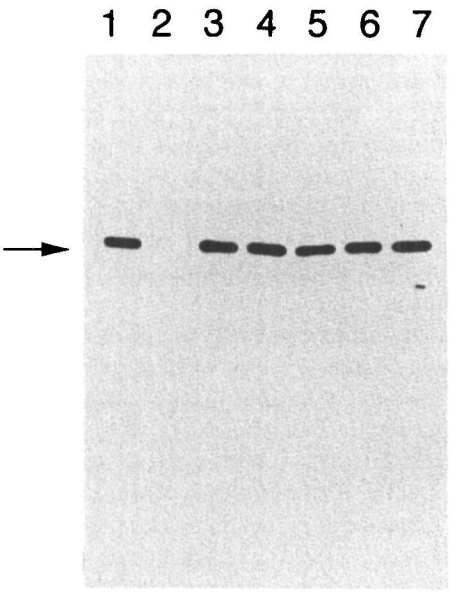

Fig. 4 Western analysis of total cell proteins. 1; wild type, 2; Fud7, 3; the transformant with wild type *psbA* and *aadA* cassette (control), 4; Ser345Cys, 5; Ser345Gly, 6; Ser345Val, 7; Ser345Phe. Total cell proteins (5 μg chlorophyll) were solubilized by incubating at 100°C in the presence of 2% SDS and 0.1 M dithiothreitol for 1 min, separated by SDS-polyacrylamide gel electrophoresis and subsequently electroblotted onto nitrocellulose filter. Proteins on the filter were hybridized by an antibody raised against D1 protein and the bands were visualized by enhanced chemiluminescence.

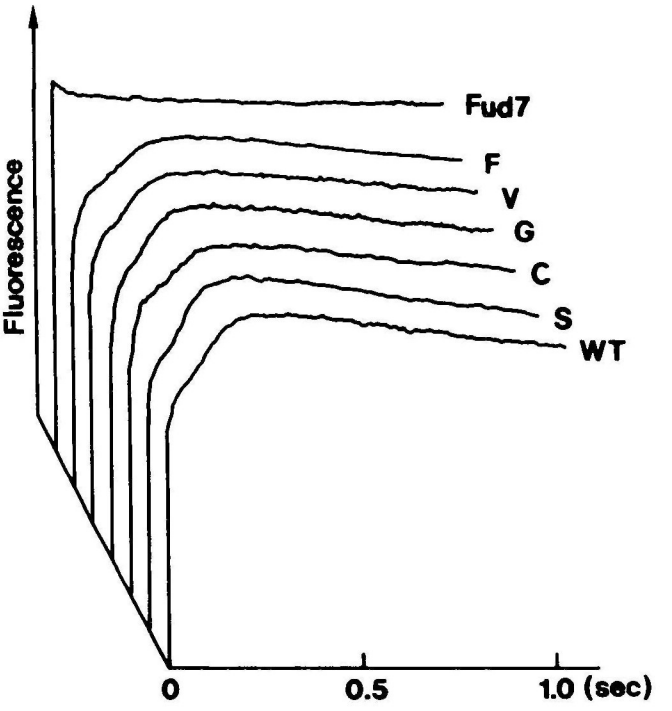

Fig. 5 Fluorescence induction transients. Cells grown in TAP culture were adapted in the dark for 10 min before measurements by Plant Efficiency Analyzer (PEA, Hansatech). WT, wild type; S, transformant with wild type *psbA* (Ser at position of 345) and *aadA* cassette (control strain); C, Ser345Cys; G, Ser345Gly; V, Ser345Val; F, Ser345Phe; Fud7, *psbA* deletion mutant.

with [^{35}S]Na_2SO_4 in the presence of cycloheximide in bright light and were subsequently chased (Fig. 7). After pulse labeling, two bands corresponding to pD1 and D1 were detected. Neither of them were detected in Fud7 as expected. Since pD1 of Ser345Val and Ser345Phe transformants migrated slightly faster than that of wild type, we employed 12.5% instead of 15% acrylamide gel in these cases for better resolution of mature and precursor forms of D1. Chase experiments revealed that pD1 is processed to D1 with a half decay time of less than 5 min in wild type. It appears that the half-decay times of pD1 in the four transformants, i.e., Ser345Gly, Ser345Cys, Ser345Val and Ser345Phe, are indistinguishable from that of wild type, within the accuracy of this experiment. Therefore, it is con-

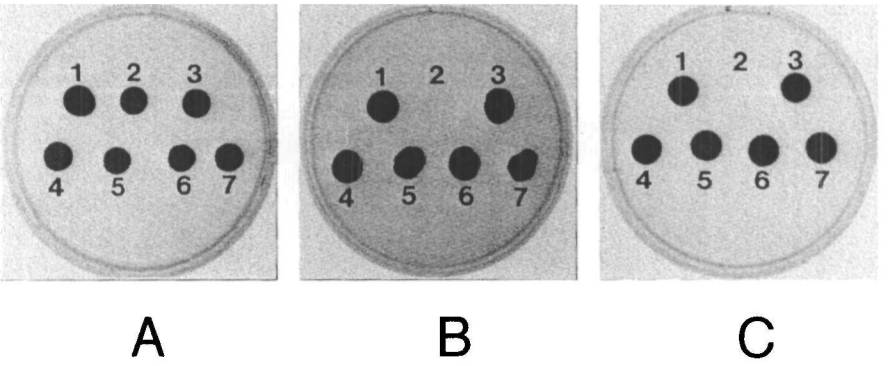

Fig. 6 Growth of the transformants under heterotrophic and photoautotrophic conditions. Aliquots of cells from 1, wild type; 2, Fud7; 3, control strain (the transformant with wild type *psbA* and *aadA* cassette); 4, Ser345Gly; 5, Ser345Cys; 6, Ser345Val; 7, Ser345Phe were spotted on agar plates. A, TAP plate in the dark; B and C, HSM plates in the light of 1,500 lux and 50,000 lux, respectively.

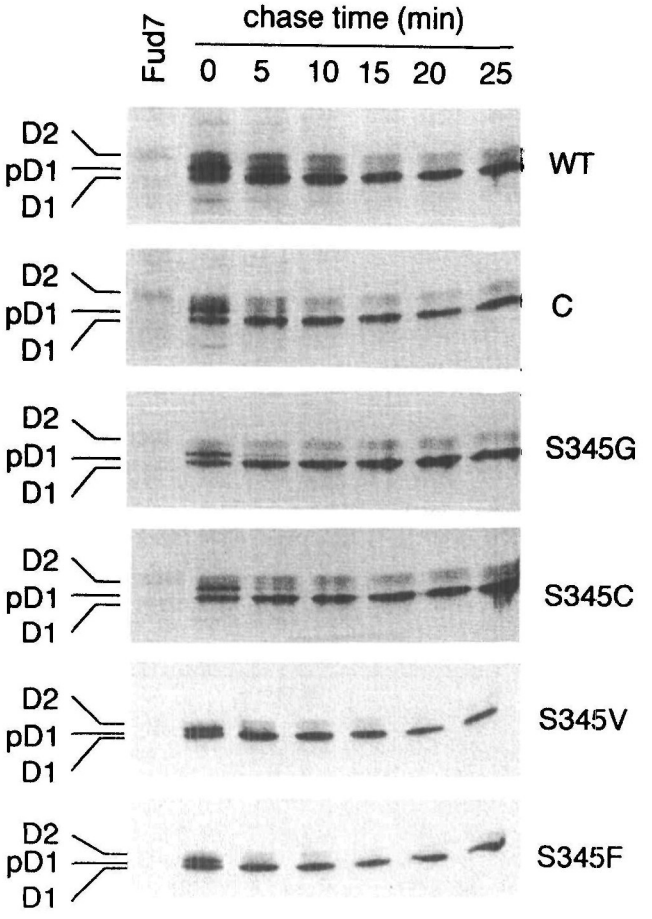

Fig. 7 Pulse and chase labeling of chloroplast-encoded proteins. Cells were grown in TAP culture at approximately 2×10^6 cells ml^{-1}, pelleted by centrifugation and suspended in TAP medium without sulfur at 2×10^7 cells ml^{-1} and incubated for 2 h. Cycloheximide (35 μg ml^{-1}) was added to cells to inhibit protein synthesis in cytosol 5 min before pulse labeling. Chloroplast encoded proteins were pulse labeled with [^{35}S]Na_2SO_4 (0.37MBq) for 3 min in bright light (10,000 lux) and then were chased in the presence of 10 mM Na_2SO_4 and chloramphenicol (200 μg ml^{-1}) for 5, 10, 15, 20 and 25 min. Labeled proteins were solubilized by 2% SDS and 0.1 M dithiothreitol at 100°C for 1 min, separated on SDS-polyacrylamide gel electrophoresis containing 6 M urea. Each lane contained 5 μg chl. The resolving gel contained 6 M urea and 15% acrylamide for WT, C (control strain; the transformant with wild type psbA and *aadA* cassette), Ser345Gly and Ser345Cys, and 12.5% acrylamide for Ser345Val and Ser345Phe.

cluded that the D1 is normally synthesized, functionally assembled in thylakoid membranes and processed even after the amino acid substitutions at +1 position.

Discussion

The transforming vector for *psbA* mutagenesis constructed in the present study contains an 11 kb chloroplast DNA fragment which corresponds to entire *psbA* gene and its flanking sequences. The DNA sequence on both ends inserted in the vector overlaps flanking regions of the deletion around the *psbA* in chloroplast genome in Fud7 strain. Therefore, the cloned *psbA* gene and its flanking regions introduced into the chloroplasts of Fud7 appears to be integrated into the chloroplast genome by homologous recombination (Whitelegge et al. 1995). Resistance to antibiotics expressed by the *aadA* cassette in the vector allows one to select directly transformants in the presence of spectinomycin.

Several site-directed mutants of the chloroplast *psbA* gene have been generated so far in *C. reinhardtii*. Przibilla et al. (1991) used as a selectable marker *psbA* gene containing a mutation which changes Ser264 to Ala and confers resistance to herbicides that inhibit electron transfer reaction between Q_A and Q_B. Transformants thus can be selected directly in the presence of herbicides under photoautotrophic condition. However, this approach introduced mutations in D1 at Ser264, together with newly designed ones, which may eventually prevent precise characterization of phenotype caused solely by mutations of interest. In addition, the selectable marker does not allow for selection of mutants which lack photoautotrophy. Another approach is to cotransform *Chlamydomonas* cells with two separate plasmids; one containing mutated *psbA* gene and the other a 16S rRNA gene conferring spectinomycin resistance (Roffey et al. 1991, Webber et al. 1995, Whitelegge et al. 1995). In this case, the screening of transformants is rather complicated; transformants are first to be selected for spectinomycin resistance and subsequently screened by DNA analysis to confirm the occurrence of desired mutation in the *psbA* gene. In addition, the mutation in 16S rRNA may affect the level of proteins in the chloroplast, since steady-state accumulation of D1 under illumination requires active protein synthesis. The transformation vector described in the present study has some advantages; it allows for direct selection of transformants with mutations in the *psbA* gene and the presence of the *aadA* cassette in the chloroplast genome appears to affect no photosynthetic performance. Similar vector containing intronless *psbA* gene with the *aadA* cassette in its downstream was also reported (Minagawa and Crofts 1994).

Using this vector, we have successfully generated chloroplast *psbA* transformants of which Ser345 has been substituted by Gly, Cys, Val and Phe in order to investigate the significance of the amino acid residue at +1 position to pD1 processing. However, the resulting four transformants were indistinguishable from wild type in terms of photosynthetic performance, steady-state accumulation of D1, and synthesis and processing rates of pD1. In particular, it is noted that there was no difference in the rate of cleavage for a small residue (Gly) and a bulky residue (Phe). A conclusion from these results is that the size and polarity of amino acid side chain at +1 position in pD1 is not crucial

for the enzymatic processing in vivo in *Chlamydomonas*.

In a cyanobacterium, *Synechocystis* sp. PCC 6803, the Ser345 has been substituted by Pro, Ala and Arg (Nixon et al. 1992). In this case, while a transformant Ser345Pro abolished processing of pD1, transformants Ser345Ala and Ser345Arg were indistinguishable from wild type in the photosynthetic growth. However, it is not yet clear whether the processing rate of pD1 in the latter two transformants is as efficient as that of wild type, since the steady-state accumulation of pD1 could not be observed in these analyses.

The enzyme responsible for cleavage of pD1 has been extracted from a green alga and higher plants (Inagakii et al. 1989, Taylor et al. 1988), partially purified (Fujita et al. 1995) and the gene encoding the protein (*ctpA*) has been cloned in a cyanobacterium, *Synechocystis* sp. PCC 6803 (Anbudurai et al. 1994, Shestakov et al. 1994) and a higher plant (Inagaki et al. 1995). It was reported that synthetic carboxyl-terminal oligopolypeptides are cleaved correctly at the processing site by the partially purified enzyme from spinach (Fujita et al. 1989). Interestingly, the rate of cleavage was largely influenced by the substitution at Ala345, i.e., the order of substitutions in terms of their effects on V_{max} was Ala, Ser, Phe, Cys>Gly>Val>>Pro (Taguchi et al. 1995). However, this in vitro analysis is inconsistent with the present results obtained in vivo in the chloroplast of *C. reinhardtii*. The discrepancy may arise from organisms used since the residue at +1 position is Ser in *C. reinhardtii* while Ala in spinach. The possibility can also be pointed out that processing rates observed by in vivo analysis may not represent the actual rate of carboxyl-terminal cleavage of pD1, since the integration of pD1 into the functional PSII complex is a complicated multi-step process as mentioned in the Introduction. However, it might be more probable that differences in the reaction condition including the size and organization of substrate are the origins of this discrepancy between in vitro and in vivo experiments. For example, the secondary and tertiary structures of small synthetic oligopolypeptides might be entirely different in solution from those of full-length pD1 integrated into thylakoid membranes. Since the sequence in extended region of pD1 is poorly conserved as mentioned, it is reasonable to speculate that the processing enzyme recognizes pD1 in its carboxyl terminal region of D1 and some other regions of mature protein, rather than the carboxy terminal extension including the residue at the +1 position.

We thank Dr. J.-D. Rochaix for Fud7 strain and *psbA* plasmid, and Dr. M. Goldschmidt-Clermont for the *aadA* cassette. This work was supported in part by the Grant in Aid for Scientific Research on Priority Areas (04273101 and 06259212), for Cooperative Research (04304004 and 05304006), General Research (06404003 and 06640842) from Ministry of Education, Science and Culture and Human Frontier Science Program.

References

Anbudurai, P.R., Mor, T.S., Ohad, I., Shestakov, G.E., Gadzhiev, A., Lind, L.K. and Pakrasi, H.B. (1994) The *ctpA* gene encodes the C-terminal processing protease for the D1 protein of the photosystem II reaction center complex. *Proc. Natl. Acad. Sci. USA* 91: 8082–8086.

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. *Plant Physiol.* 24: 1–15.

Bennoun, P., Spierer-Herz, M., Erickson, J., Girard-Bascou, J., Pierre, Y., Delosme, M. and Rochaix, J.-D. (1986) Characterization of photosystem II mutants of *Chlamydomonas reinhardtii* lacking the *psbA* gene. *Plant Mol. Biol.* 6: 151–160.

Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B. and Sanford, J.C.(1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* 240: 1534–1538.

Ellis, J.R. (1981) Chloroplast proteins: synthesis, transport, and assembly. *Annu. Rev. Plant. Physiol.* 32: 111–137.

Erickson, J.M., Rahire, M. and Rochaix, J.-D. (1984) *Chlamydomonas reinhardtii* gene for the 3,200 mol wt protein of photosystem II contains four large introns and is located entirely within the chloroplast inverted repeat. *EMBO J.* 3: 2753–2762.

Fujita, S., Inagaki, N., Yamamoto, Y., Taguchi, F., Matsumoto, A. and Satoh, K. (1995) Identification of the carboxyl-terminal processing protease for the D1 precursor protein of the photosystem II reaction center of spinach. *Plant Cell Physiol.* 36: 1169–1177.

Fujita, S., Inagaki, N., Ono, T., Inoue, Y. and Satoh, K. (1989) Cleavage of a synthetic COOH-terminal oligopeptide of D1 precursor protein by a purified processing enzyme. *FEBS Lett.* 255: 1–4.

Goldschmidt-Clermont, M. (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *chlamydomonas*. *Nucl. Acids Res.* 19: 4083–4089.

Gorman, D.S. and Levine, R.P.(1965) Cytochrome *f* and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardti*. *Proc. Natl. Acad. Sci. USA* 54: 1665–1669.

Inagaki, N., Fujita, S. and Satoh, K. (1989) Solubilization and partial purification of a thylakoidal enzyme of spinach involved in the processing of D1 protein. *FEBS Lett.* 246: 218–222.

Inagaki, N., Yamamoto, Y., Mori, H. and Satoh, K. (1995) Carboxyl-terminal processing protease for the D1 precursor protein: cloning and sequencing of the spinach cDNA. *Plant Mol. Biol.* (in press).

Johanningmeier, U. and Heiss, S. (1993) Construction of a *Chlamydomonas reinhardtii* mutant with an intronless psbA gene. *Plant Mol. Biol.* 22: 91–99.

Karabin, G.D., Farley, M. and Hallick, R.B. (1984) Chloroplast gene for M_r 32,000 polypeptide of photosystem II in *Euglena gracilis* is interrupted by four introns with conserved boundary sequences. *Nucl. Acids. Res.* 12: 5801–5812.

Keller, M. and Stutz, E. (1984) Structure of the *Euglena gracilis* chloroplast gene (*psbA*) coding for the 32-kDa protein of Photosystem II. *FEBS Lett.* 175: 173–177.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lers, A., Heifetz, P.B., Boynton, J.E., Gillham, N.W. and Osmond, C.B. (1992) The carboxyl-terminal extension of the D1 protein of photosystem II is not required for optimal photosynthetic performance under CO_2- and light-saturated growth conditions. *J. Biol. Chem.* 267: 17494–17497.

Marder, J.B., Goloubinoff, P. and Edelman, M. (1984) Molecular architecture of the rapidly metabolized 32-kilodalton protein of photosystem II: indication for COOH-terminal processing of a chloroplast membrane polypeptide. *J. Biol. Chem.* 259: 3900–3908.

Mattoo, A.K., Marder, J.B. and Edelman, M. (1989) Dynamics of the photosystem II reaction center. *Cell* 56: 241–246.

Minagawa, J. and Crofts, A.R. (1994) A robust protocol for site-directed mutagenesis of the D1 protein in *Chlamydomonas reinhardtii*: a PCR-spliced *psbA* gene in a plasmid conferring spectinomycin resistance was introduced to a *psbA* deletion strain. *Photosynth. Res.* 42: 121–131.

Minami, E. and Watanabe, A. (1985) Detection of a precursor polypeptide of the rapidly synthesized 32,000-dalton thylakoid protein in spinach chloroplast. *Plant Cell Physiol.* 26: 839–846.

Nanba, M. and Satoh, K. (1987) Isolation of a photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559. *Proc. Natl. Acad. Sci. USA* 84: 109–112.

Nixon, P.J., Trost, J.T. and Diner, B.A. (1992) Role of the carboxy terminus of polypeptide D1 in the assembly of a functional water-oxidizing manganese cluster in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: assembly requires a free carboxyl group at C-terminal position 344. *Biochemistry* 31: 10859–10871.

Pfister, K., Steinback, K.E., Gardner, G. and Arntzen, C.J. (1981) Photoaffinity labeling of an herbicide receptor protein in chloroplast membranes. *Proc. Natl. Acad. Sci. USA* 78: 981–985.

Przibilla, E., Heiss, S., Johanningmeier, U. and Trebst, A. (1991) Site-specific mutagenesis of the D1 subunit of photosystem II in wild-type *Chlamydomonas. Plant Cell* 3: 169–174.

Reisfeld, A., Mattoo, A.K. and Edelman, M. (1982) Processing of a chloroplast-translated membrane protein in vivo. Analysis of the rapidly synthesized 32,000-dalton shield protein and its precursor in *Spirodela oligorrhiza. Eur. J. Biochem.* 124: 125–129.

Rochaix, J.-D. (1978) Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardtii. J. Mol. Biol.* 126: 597–617.

Roffey, R., Golbeck, J.H., Hille, C.R. and Sayre, R.T. (1991) Photosynthetic electron transport in genetically altered photosystem II reaction centers of chloroplast. *Proc. Natl. Acad. Sci. USA* 88: 9122–9126.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schrader, S. and Johanningmeier, U. (1992) The carboxy-terminal extension of the D1-precursor protein is dispensable for a functional photosystem II complex in *Chlamydomonas reinhardtii. Plant Mol. Biol.* 19: 251–256.

Shestakov, S.K., Anbudurai, P.R., Stanbekova, G.E., Gadzhiev, A., Lind, L.K. and Pakrasi, H.B. (1994) Molecular cloning and characterization of the *ctpA* gene encoding a carboxyl-terminal processing protease. Analysis of a spontaneous photosystem II-deficient mutant of the cyanobacterium *Synechocystis* sp. PCC6803. *J. Biol. Chem.* 269: 19354–19359.

Svensson, B., Vass, I. and Styring, S. (1991) Sequence analysis of the D1 and D2 reaction center proteins of photosystem II. *Z. Naturforsch.* 46c: 765–776.

Taguchi, F., Yamamoto, Y. and Satoh, K. (1995) Recognition of the structure around cleavage site by the carboxyl-terminal processing protease for D1 precursor protein of photosystem II reaction center. *J. Biol. Chem.* 270: 10711–10716.

Takahashi, M., Shiraishi, T. and Asada, K. (1989) COOH-terminal residues of D1 and the 44 kDa CPa-2 at spinach photosystem II core complex. *FEBS Lett.* 240: 6–8.

Takahashi, Y., Goldschmidt-Clermont, M., Soen, S.-Y., Franzén, L.G. and Rochaix, J.-D. (1991) Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilizes photosystem I. *EMBO J.* 10: 2033–2040.

Takahashi, Y., Matsumoto, H., Goldschmidt-Clermont, M. and Rochaix, J.-D. (1994) Directed disruption of the *Chlamydomonas* chloroplast *psbK* gene destabilizes the photosystem II reaction center complex. *Plant Mol. Biol.* 24: 779–788.

Takahashi, Y., Nakane, H., Kojima, H. and Satoh, K. (1990) Chromatographic purification and determination of the carboxy-terminal sequences of photosystem II reaction center proteins, D1 and D2. *Plant Cell Physiol.* 31: 273–280.

Taylor, M.A., Packer, J.C.L. and Bowyer, J.R. (1988) Processing of the D1 polypeptide of the photosystem II reaction center and photoactivation of a low-fluorescence mutant (LF-1) of *Scenedesmus obliquus. FEBS Lett.* 237: 229–233.

Webber, A.N., Bingham, S.E. and Hyeonmoo, L. (1995) Genetic engineering of thylakoid protein complexes by chloroplast transformation in *Chlamydomonas reinhardtii. Photosynth. Res.* 44: 191–205.

Weeks, D.P., Beerman, N. and Griffith, O.M. (1986) A small-scale five-hour procedure for isolating multiple samples of CsCl-purified DNA: application to isolations from mammalian, insect, higher plant, algal, yeast, and bacterial sources. *Anal. Biochem.* 152: 376–385.

Whitelegge, J., Koo, D., Diner, B.A., Domian, I. and Erickson, J.M. (1995) Assembly of the photosystem II oxygen-evolving complex is inhibited in psbA site-directed mutants of *Chlamydomonas reinhardtii*; Aspartate 170 of the D1 polypeptide. *J. Biol. Chem.* 270: 225–235.

(Received September 22, 1995; Accepted December 25, 1995)