THE CHLOROPLAST GENE EXPRESSION IN *CHLAMYDOMONAS REINHARDTII*

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To Keizo Ishikura, Mie Ishikura,

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for their understanding and devotion.

CONTENTS

ABBREVIATIONS

kb	kilobase
bp	base pair
nt	nucleotides
kDa	kilodalton
min	minute
hr	hour
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
Tris	tris(hydroxymethyl)methylglycine
SDS	sodium dodecyl sulfate
TAP medium	Tris acetate phosphate medium
M medium	Tris-minimal medium
PCR	polymerase chain reaction
SDS-PAGE	SDS polyacrylamide gel electrophoresis
cp genome	chloroplast genome
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
LSU	RuBisCO large subunit
CES process	a control by the epistasy of synthesis
UTR	untranslated region
C. reinhardtii	*Chlamyodomonas reinhardtii*
E. coli	*Escherichia coli*

CHAPTER I

Introduction

The features of the chloroplast

Chloroplast is one of the most distinct features in plant cells. This organelle promotes vital reactions for plants, such as photosynthesis, carbon fixation, amino acids and sulfur metabolism, lipid biosynthesis, and nitrate reduction. The chloroplast contains its own genome in the form of double-stranded circular DNA molecules. The complete nucleotide sequences of the genome have been determined in several species, such as tobacco (Shinozaki et al., 1986), liverwort (Ohyama et al., 1986), rice (Hiratsuka et al., 1989), maize (Maier et al., 1995), and *Chlorella vulgaris* (Wakasugi et al., 1997). The great majority of the genome, representing all of the major land plants and algae, vary in size from 120 kb to 200 kb (Mayfield et al., 1995; Sugita and Sugiura, 1996; Sugiura, 1992). The sequence contains more than 100 functional genes encoding rRNAs and tRNAs, as well as chloroplast-localized proteins. The latter can be grouped into proteins involved in photosynthesis and carbon fixation, and those for the chloroplast gene expression itself, such as ribosomal proteins, translation factors, and transcription apparatus. The comparison of the chloroplast genome sequence shows prokaryotic features. For example, the promoters have considerable similarity to typical bacterial promoters of the *Escherichia coli* σ^{70} or *Bacillus subtilis* σ^{43}. Putative chloroplast promoters generally contain hexanucleotide resembling bacterial sequences -10 (TATAAT) and -35 (TTGACA) upstream of the transcription start site. The chloroplast genes also share the homology with the nuclear sequences for the bacterial α, β, β' subunits of RNA polymerases. Similarly, the regulation factor for transcription is homologous to the bacterial σ factor (Tanaka et al., 1996; Troxler et al., 1994). Moreover, as observed in bacterial genes, some chloroplast genes are organized into polycistronic transcription units both in higher plants and in green algae. As well as in transcription, prokaryotic features are found in the chloroplast translation machinery. The chloroplast contains 70S ribosomes, composed of 50S and 30S ribosomal subunits, and fMet-initiator tRNA. In addition, 5'-terminus of mRNAs is not capped, and DNA sequence of the *16S rRNA*, encoding 16S ribosomal RNA, resembles to the bacterial counterpart (Dron et al., 1982). The ribosome binding site, or Shine-Dalgarno sequence, is also identifiable in some chloroplast genes. In spite of these similarities, differences can be found between the chloroplast and bacteria. For instance, one of the core in the promoter, the -35 element is not always necessary. An additional transcription activity, which is independent from the core promoter, may also exist (Kapoor et al., 1997). Moreover, mRNAs from chloroplast genes live longer than bacterial mRNAs, and even ribosome-free mRNAs can be observed. Several transcripts are altered by RNA editing to be functional as mitochondorial mRNAs (Hanson et al., 1996). In addition to these differences, the chloroplast is semiautonomous and the gene

expression is controlled by products of nuclear genes (Rochaix, 1992). These evidence imply that the chloroplast may have distinct features in its gene expression.

Chlamydomonas reinhardtii as a model system for the study of the chloroplast gene expression

In general, the chloroplast gene expression has been examined in tobacco, spinach, barley, and a green alga, *Chlamydomonas reinhardtii.* Among these species, *C. reinhardtii* grows relatively fast with approximately 5 to 6 hours for one generation, and can be grown either in liquid culture or as individual colonies on agar plates (Harris, 1985). This organism is a unicellular so that the homologous cell population can be obtained. The chloroplast of this alga is analogous to those of higher plants. Because of its cell permeability, newly synthesized transcripts and proteins can also be labeled *in vivo* (Baker et al., 1984; Takahashi et al., 1994). In addition, photosynthetic mutants can be maintained in a medium containing acetic acid as a carbon source. Furthermore, the chloroplast transformation, by a particle bombardment, has been established only in *C. reinhardtii* (Boynton et al., 1988) except in tobacco (Svab et al., 1990). *C. reinhardtii* has a single chloroplast with less than 100 genome copies so that the homoplasmicity can be achieved with ease in the transformation. On the other hand, tobacco is multi-cell and each cell contains a total of approximately 10,000 genome copies in 100 chloroplasts, making the plant difficult to obtain the homoplastomic state (Maliga and Nixon, 1998). Because of the advantage in the transformation and the characters described above, *C. reinhardtii* has the credentials of a good model system for the study of the chloroplast gene expression.

Mutants of *C. reinhardtii*

Both chloroplast and nuclear mutants, in which the expression of chloroplast-encoded components of photosystem I, photosystem II, ATP synthase, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), or the photosynthetic electron transport chain is altered, have been isolated in *C. reinhardtii* (Rochaix, 1992). These mutations affect the genes for transcription, the splicing and stability of mRNA, and protein synthesis. Also, a number of transformants were generated in which mutations were introduced into the genes of interest by the chloroplast transformation. From analyses of these mutants, several *cis*-elements and *trans*-acting factors have been determined to regulate the chloroplast gene expression.

Reporter analysis

Using the particle bombardment, a transgene can be stably introduced into the chloroplast, which makes the reporter analysis available (Blowers et al., 1989). In *C. reinhardtii*, *E. coli.* genes *uidA* and *aadA*, encoding β-glucuronidase (GUS) (Jefferson et al., 1986) and aminoglucoside adenine transferase (Hollingshead and Vapnek, 1985), respectively, have been

introduced to the chloroplast as reporters (Choquet et al., 1998; Sakamoto et al., 1993). Several regulatory sequences have been determined by the fusion of a fragment from genomic sequence and a reporter gene.

The mission of this study

Although mutants gave us fruitful results, an interruption of the expression in the chloroplast gene which encodes one subunit may cause attenuation of other subunits from the same complex described as a control by the epistasy of synthesis (CES process) (Choquet et al., 1998). In addition, mutations usually disturb photosynthesis, and subsequently, a global gene expression in the chloroplast could be changed. In such case, the mutant may not show the actual gene function.

Another problem for the analysis of the chloroplast gene expression is that most studies have focused on only one or two, but not on every levels of the expression. Chloroplast genes are regulated at the level of transcription, post-transcription, translation, and post-translation (i.e., protein modification), as is the case for the gene in prokaryotes or eukaryotes (Fig. 1-1). Because how much each level contributes to the over-all gene expression or whether each level influences to others is not known, each level should be analyzed, and we should understand the chloroplast gene expression as a sequence of each process.

Here, the *uidA* reporter gene which contains a portion of the chloroplast gene was introduced into the *C. reinhardtii* chloroplast genome. Because the reporter gene is believed not to disturb endogenous genes, the analysis may reflect the actual gene expression in the chloroplast. In addition to this advantage, the expression of *chimeric-uidA* can be monitored at the level of transcription (transcription activity), post-transcription (accumulation and stability of the transcript), and translation (accumulation of GUS protein). In this thesis, I describe the chloroplast gene expression in the followings;

(1) Effects of the promoter and 5'-untranslated region on the chloroplast gene expression.

(2) Effects of the 5'-coding sequence on the chloroplast gene expression.

(3) Effect of translation on mRNA stability.

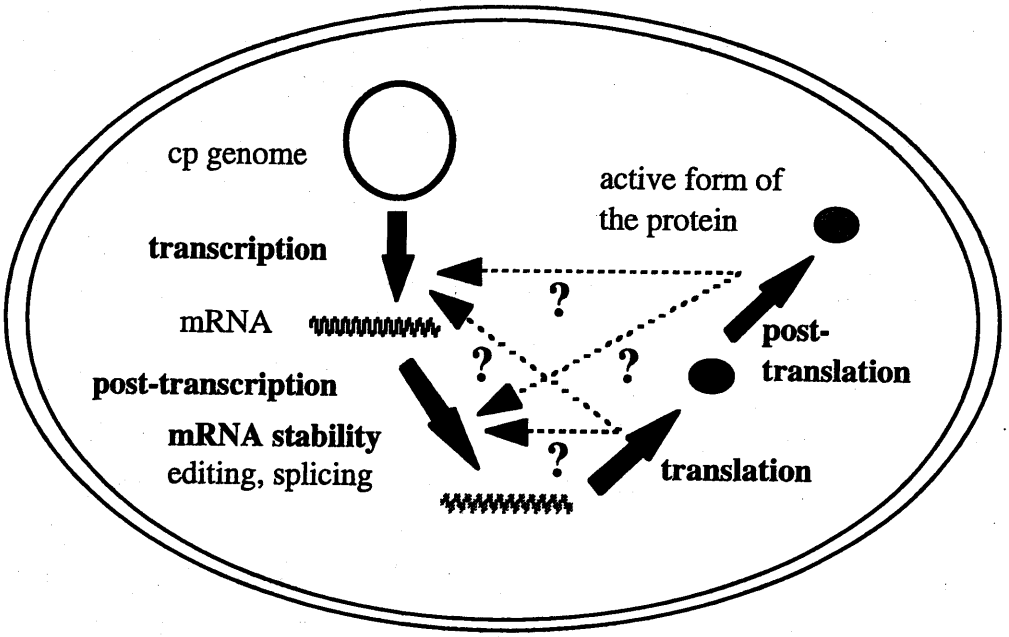

Figure 1-1. Gene expression in the chloroplast (cp). The chloroplast contains its own genome and gene expression system. The chloroplast genes are regulated at each level of transcription, post-transcription, translation, and post-translation. Each level contributes to the gene expression. How each level influences to others is not known.

CHAPTER II

Effects of the promoter and 5'-untranslated region on the chloroplast gene expression

Introduction

One of the steps regulates the gene expression in the chloroplast is transcription. The alignment of sequences from chloroplast putative promoter regions in higher plants shows that chloroplast genes contain consensus prokaryotic -35 (TTGACA) and -10 (TATAAT) elements. However, the -35 element is not always identifiable in higher plants (Mayfield et al., 1995). Also, seven genes in eight chloroplast genes contain a TATAATAT motif in the -10 region, while six genes in those eight genes lack the typical conserved -35 element in *C. reinhardtii* (Fig. 2-1). Based on the number of deletion analyses *in vitro* and *in vivo*, most promoters of chloroplast genes simply require the -10 consensus, and in some cases, the additional -35 consensus sequence (Blowers et al., 1993; Gruissem and Zurawski, 1985). Some upstream sequence of the core elements also enhance transcription. In rice, the deletion of the upstream sequence, from -546 to -100 from the transcription start point, resulted in 4 to 5-fold decrease in the transcription activity of *psbD-psbC* operon, encoding photosystem II reaction center protein D2 and chlorophyll a-binding protein CP43 (To et al., 1996). Additional deletion from -99 to -40 showed further 2-fold decrease. Moreover, a downstream of the consensus sequence appears to enhance transcription. Deletion series of the *atpB* gene, encoding β subunit of ATP synthase, showed the downstream sequences, up to +55 from the transcription start, were required for the basal level of transcription in *C. reinhardtii* (Klein et al., 1992). A chimeric reporter gene containing only the putative promoter sequence of the *rbcL*, encoding the large subunit of RuBisCO, was transcribed merely 15% as actively as the endogenous *rbcL* gene (Klein et al., 1994). These results indicate that the downstream of the -10 consensus affects transcription activity.

The gene expression is regulated not only at the level of transcription, but also at the level of post-transcription. The 5'-untranslated region (5'-UTR) plays an important role in post-transcription, and one of roles is to regulate mRNA stability. In *C. reinhardtii*, an eight nucleotide (nt) element at the 5'-end of the transcript from the *petD*, encoding IV subunit of the cytochrome b_6/f complex, is necessary for the RNA processing and stability *in vivo* (Higgs et al., 1997; Sakamoto et al., 1994; Sakamoto et al., 1994) . The *rbcL* 5'-UTR fused to a reporter gene could respond to light, resulting in the light-induced degradation of the *rbcL* mRNA (Salvador et al., 1993). In addition, several nuclear mutants of *C. reinhardtii* have been isolated that fail to accumulate individual chloroplast mRNAs, despite having the wild-type levels of transcription rate (Rochaix, 1992). In one of such mutants, nac2-26, the transcript from the

psbD was unstable and the accumulation of the transcript was reduced (Kuchka et al., 1989). The *psbD* 5'-UTR fused to a reporter gene accumulated the transcript in the wild-type but not in nac2-26, suggesting the gene product mutated in nac2-26 interacts with the *psbD* 5'-UTR to stabilize the mRNA (Nickelsen et al., 1994) . From the analysis of the mutant F16, the *petD* 5'-UTR appeared to interact with a nuclear-encoded factor that is necessary for the mRNA accumulation (Drager et al., 1998) . In case of a higher plant, the tobacco *rbcL* 5'-UTR is responsible for stabilizing the *rbcL* mRNA in the dark (Shiina et al., 1998).

Moreover, several *C. reinhardtii* mutants implicate the 5'-UTR plays a role in translation. A chloroplast mutant, Fud34, does not synthesize the subunit III of photosystem II protein, encoded by the *psbC* gene (Rochaix et al., 1989). The mutation was mapped within the 5'-UTR of the *psbC*. The deletion analysis showed that a 97 nt region located in the middle of the *psbC* 5'-UTR is required for translation initiation (Zerges et al., 1997). Also, two regions of the *petD* 5'-UTR appeared to act as positive elements for translation (Sakamoto et al., 1994). Another nuclear mutants show that the *trans*-acting factors, located on the loci TBC1, TBC2, and TBC3, interact to the *psbC* 5'-UTR (Zerges et al., 1997; Zerges and Rochaix, 1994). In addition, a nuclear factor, located on TAB1 locus, may bind to the 5'-UTR of *psaB*, encoding a polypeptide of the photosystem I reaction center, and initiate protein synthesis (Stampacchia et al., 1997). Similarly, a minimum set of six chloroplast 5'-UTR-binding proteins (81, 62, 56, 47, 38, and 15 kDa) were observed in all cell types of *C. reinhardtii*, and these proteins appeared to regulate translation (Hauser et al., 1996).

In this chapter, I describe the influence of the promoter and 5'-UTR to the gene expression in the *C. reinhardtii* chloroplast. Using the *uidA* reporter gene, the expression was examined at the levels of transcription, post-transcription, and translation. First, *chimeric-uidA* genes, each carrying a coding sequence of the *uidA*, and 3'-untranslated region (3'-UTR) from the *rbcL*, were constructed. Each chimeric gene also contained a putative promoter and a full length 5'-UTR of the *rbcL*, *psbA*, or the *atpA*. The *psbA* and *atpA* encodes D1 protein in photosystem II and a subunit of ATP synthase, respectively. These three genes were selected for several reasons: (1) Sequences and transcription start sites were already determined for all three genes. The *rbcL* contains both -35 and -10 elements, while the *psbA* or *atpA* only contains the -10 element (Fig. 2-1). (2) The *rbcL* and *psbA* are monocistronic, while the *atpA* belongs in the polycistronic unit. (3) Transcription activities of the three genes were determined (Blowers et al., 1990). Among the eight genes, the *rbcL* and *psbA* were most actively transcribed except *16S rRNA* encoding 16S ribosomal RNA. Transcription activities of the *rbcL* and *psbA* were 31.2% and 13.2%, relative to the activity of the *16S rRNA*. On the other hand, the relative activity of the *atpA* was only 1.8% (Table 2-1). (4) These candidates were well examined at the level of post-transcription. For example, the level of the *atpA* transcript showed circadian oscillation, while levels of the *rbcL* and *psbA* were unchanged in the light-dark cycle (Salvador et al., 1993). The result indicated the different mechanism for mRNA accumulations may exist,

and the mechanism was depending on the gene. Both the *rbcL* and *psbA* transcripts are known as the most abundant mRNAs in the chloroplasts among many species. (5) Products translated from the *rbcL* and *psbA* are two major proteins in the chloroplast. On the other hand, *in vivo* pulse study showed that the translation activity of the *atpA* was significantly higher than the rates of the *rbcL* and *psbA* (Delepelaire, 1983).

Chimeric genes, containing the sequences of the *rbcL*, *psbA*, and the *atpA*, were introduced into the chloroplast genome of *C. reinhardtii* by the particle bombardment. Subsequently, stable chloroplast transformants RG, PG, and AG were acquired. RG, PG, or AG harbored the promoter and 5'-UTR from the *rbcL*, *psbA*, or the *atpA*, respectively. Then, the expression of chimeric genes in transformants were examined. Results showed that promoters and 5'-UTRs alone did not account for the expression of endogenous genes in the chloroplast of *C. reinhardtii*.

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                -40          -30          -20          -10           1
                 *            *            *            *            *
atpB       TCTAA AGATGAGTAC AATGTTTTGG AATATTTATA TAATATATTA A
atpA       CAATC AATTTATAAA TATATTTATT ATTATGCTAT AATATAAATA C
psaA       TAATT TGTAAACCAA TAAAAAATAT ATTTATGGTA TAATATAACA T
psbD       AAATG CTTATTTTTA ATTTTATTTT ATATAAGTTA TAATATTAAA T
psbA       ATGTG CTAGGTAACT AACGTTTGAT TTTTTGTGGT ATAATATATG T
petA       TGCAT GAACTATGCT TTATTTGCTA AAAAAAAGAT ATAATATATG T
rbcL       TTGCT AGTTTACATT ATTTTTTATT TCTAAATATA TAATATATTT A
16S rRNA   AAAAT AAAAATTTGA CAAAAAAAAA TAAAAAAGTT AAATTAAAAA C
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Figure 2-1. Sequences upstream of the transcription initiation site in chloroplast genes of *C. reinhardtii*. Putative -10 and -35 motifs are underlined (Klein et al., 1992).

Table 2-1. Relative activity of transcription for each chloroplast gene (Blowers et al., 1990).

gene [a)]	relative transcription rate (%)
rrn	100.0
psbA	13.2
rbcL	31.2
psaB	1.3
atpA	1.8
atpB	4.3
tufA	3.0
rpl16	4.9

a) *rrn* represents *16S rRNA* gene. The *tufA* and *rpl16* both encode translational apparatus.

Materials and Methods

Strain and culture conditions

C. reinhardtii wild-type 137c, obtained from Dr. M. Goldschmidt-Clermont (University of Geneva), and transformants were grown in a Tris acetate phosphate medium (TAP) or a Tris-minimal medium (M) (Rochaix et al., 1988) under continuous light (1,000 lx) at 28°C. A *rbcL* disruptant DEVL was grown in a TAP medium in the dark. Where necessary, the medium were solidified with 2 % agar and supplemented with spectinomycin. Cells were harvested at 2×10^6 cells/ml following growth in a liquid medium for all RNA isolations and protein isolations except *in vivo* labeling study.

DNA constructs

Recombinant DNA plasmids were prepared by conventional procedures (Sambrook et al., 1989). Bacterial host was *E. coli* DH5α.

To abolish the *Sph*I site, plasmid vector pUC19 was digested with *Sph*I, filled-in with T4 DNA polymerase, and ligated to obtain plasmid pUC19dS. A 3.9 kb *Eco*RI-*Bam*HI fragment from plasmid R15 (Dron et al, 1982) containing the *C. reinhardtii rbcL* and *psaB* genes was inserted into *Eco*RI-*Bam*HI digested pUC19dS to obtain plasmid pUCEB (Fig. 2-3A). A 1.9 kb *Eco*RV-*Sma*I fragment containing the *aadA* expression cassette (Goldschmidt-Clermont, 1991) was inserted into pUCEB partially digested with *Hpa*I to obtain plasmid pUCEBaadA. The *aadA* gene confers *Chlamydomonas* cells resistant to spectinomycin. To delete the portion of the *rbcL* coding sequence, *Eco*RV-*Cla*I digested pUCEBaadA was filled-in with Klenow fragment following by ligation, resulting the plasmid pUCEBdrbcL (Fig. 2-3A).

The coding sequence of the β-glucuronidase gene (*uidA*) was amplified by the polymerase chain reaction (PCR) using primers GUS5 and GUS3 (Table 2-2) and plasmid pBI101 (Jefferson et al., 1986) as a template. Amplified fragment was inserted into pT7Blue T-Vector (Novagen) to obtain plasmid pT7GUS. A 1.8 kb *Nco*I-*Sph*I fragment from the pT7GUS was inserted into the *Nco*I-*Sph*I digested pUCEBaadA to obtain plasmid pUCEBGUS. The *Nco*I site contains the translation initiation site of the *uidA* gene.

For the PCR amplification of a 5'-sequence containing the promoter and 5'-UTR from the *rbcL*, *psbA*, and *atpA* genes, whole genome was isolated from *C. reinhardtii* wild-type 137c (Rochaix et al., 1988) and used as a template. PCR primer pairs were RBCL5 and RBCL3 for the 5'-*rbcL*, PSBA5 and PSBA3 for the 5'-*psbA*, and ATPA5 and ATPA3 for the 5'-*atpA*, respectively (Table 2-2). These 5'-sequences were subcloned into pT7Blue T-Vector, and then excised with *Cla*I and *Nco*I. A 0.37 kb 5'-*rbcL*, 0.25 kb 5'-*psbA*, and a 0.58 kb 5'-*atpA* *Cla*I-*Nco*I fragments were introduced into *Cla*I-*Nco*I digested pUCEBGUS. Resulting plasmids pGrbcL, pGpsbA, and pGatpA carry the *rbcL-uidA*, *psbA-uidA*, and the *atpA-uidA*, respectively (Fig. 2-3B). Comparing with the database, the 5'-sequences showed several

nucleotide substitutions. These substitutions were reconfirmed by independent PCR products. Our sequences were registered in DDBJ, EMBL and GenBank nucleotide sequence database with following accession numbers; AB016252 for the 5'-*rbcL*, AB016253 for the 5'-*psbA*, and AB016254 for the 5'-*atpA*, respectively.

For the analysis of a specific chloroplast mRNA, the coding sequences of the chloroplast genes were amplified by PCR. Primer pairs were 16S5 and 16S3 for a 0.5 kb fragment of the *16S rRNA*, PF and PR for a 1.5 kb coding sequence of the *rbcL*, PSBA5 and PSBA30 for a 0.3 kb exon 1 of the *psbA*, and ATPAC5 and ATPAC3 for a 1.4 kb fragment of the *atpA* (Table 2-2). Each PCR product was subcloned into the plasmid vector pUC19. Resulting plasmids were designated pUC16S, pUCrbcL, pUCpsbA, and pUCatpA, respectively. A 1.8-kb *Pst*I-*Bam*HI fragment from pT7GUS was cloned into pUC19 to create plasmid pUCGUS. Where necessary, plasmids were digested with restriction enzymes as probes for the mRNA hybridization.

Chloroplast transformations

To obtain the *rbcL* disruption mutant DEVL, *C. reinhardtii* wild-type cells were plated onto the TAP-agar medium containing 100 μg/ml spectinomycin at a density of approximately 1×10^7 cells/90 mm petri plate. Gold particles (Ø 1 μm) coated with the plasmid pUCEBdrbcL were delivered into *Chlamydomonas* cells using a Bio-Rad PDS 1000He Biolistic gun at 1100 p.s.i. After incubating in the dark for one week, the transformant colonies were subcultured on TAP-spectinomycin plates and the homoplasmic state was repeatedly confirmed by PCR.

To obtain transformants harboring the *chimeric-uidA*, the nonphotosynthetic mutant DEVL cells were plated onto the M-agar medium at a density of approximately 1×10^7 cells/90 mm petri plate. Then, gold particles coated with plasmids which carry *chimeric-uidAs* were delivered into DEVL cells (Fig. 2-2). After incubation under the light for two weeks, photosynthetic growing transformants were subcultured on M plates. *Chimeric-uidA* insertions and the homoplasmicity of transformants were confirmed after no less than three consecutive cultures.

PCR

Twenty-five cycles of amplification were performed in a Perkin-Elmer thermal cycler. The amplification cycle was programmed with 1 min of denaturing at 95°C, 2 min of annealing at 55°C, and 2 min of extension at 72°C. The amplified products were analyzed by electrophoresis in a 1% agarose gel.

Preparation of the crude extract from *C. reinhardtii* cells

Cells grown in the TAP or M medium were harvested by centrifugation at 2,000 × *g* for 10 min at 4°C. The pellet was resuspended in 1.3 ml of GUS lysis buffer [50 mM

NaH_2PO_4/Na_2HPO_4 (pH 7.0), 10 mM EDTA, and 10 mM 2-mercaptoethanol], followed by sonication. The lysate was centrifuged at 100,000 × *g* for 1 hr at 4°C, and the supernatant was used for the subsequent experiments.

GUS assays

The fluorescence assay of GUS activity was performed with 4-methylumbelliferyl glucuronide as a substrate (Jefferson et al., 1986). GUS activity was described as nanomoles of methylumbelliferone per minute per mg of protein.

Western analysis of GUS protein

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5% acrylamide gel (Laemmli, 1970), and electroblotted to Hybond-P membrane (Amersham). Immunoblotting was subjected with the rabbit polyclonal antibody raised against *E. coli* GUS protein (Molecular probe, #A-5790) and a goat anti-rabbit IgG alkaline phosphatase conjugate, following the manufacture manual (Promega). Protein was measured by the Coomassie blue dye-binding method (Bradford, 1976).

RNA isolation

For northern analysis, cells were lysed by extensive grinding with a pestle and a mortar in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl, 5 mM EDTA, 2 mM aurin tricarboxylic acid, 2% SDS, 2% Na-triisopropylnaphtalene sulfonate, and 12.8 mM 2-mercaptoethanol in liquid nitrogen. The 1.4 ml of 3 M KCl was added to the homogenate and stored on ice for 15 min. After centrifugation at 2,000 × *g* for 20 min at 4°C, 7.3 ml of 10 M LiCl were added to the supernatant and stored on ice for 2 hr. Insolubilized RNA was precipitated by centrifugation at 30,000 × *g* for 25 min at 4°C, dissolved in 4 ml of water, and then extracted with phenol/chloroform (24:1) and chloroform to remove proteins in the RNA solution. The total cellular RNA was precipitated with ethanol and dissolved in 50 μl of water. For *in vivo* labeling study, total RNA was extracted from the cell with FastRNA kit-RED (BIO 101), following the manufacture manual except for extracting with phenol, phenol/chloroform (24:1), and with chloroform.

Northern analysis

Individual steps of the procedure were followed essentially as already described (Sambrook et al., 1989). Total RNA (5 μg) was separated in a 1.5% agarose/formaldehyde gel, and transferred onto Hybond-N^+ membrane (Amersham). For hybridization, DNA fragments as gene specific probes were removed from vectors (described above) with restriction enzymes, following by gel purification, and were labeled with [α-^{32}P]dCTP and using BcaBest labeling kit (Takara). Prehybridization and hybridization were performed in Church hybridization buffer

at 65°C (Church and Gilbert, 1984). After the hybridization, the filter was washed with 2 × SSC for 5 min at room temperature, followed by several washes at 65°C. Then the filter was exposed to a X-ray film with an intensifying screen at -80°C. For the determination of the signal intensity, the filter was exposed to an imaging plate (Fuji Photo Film), and signals were quantified by a bio-imaging analyzer BAS 2000 (Fuji Photo Film).

In vivo RNA labeling

C. reinhardtii grown in the TAP medium as a preculture, was inoculated to 300 ml of low-phosphate TAP medium in which phosphate concentration was reduced from 1 mM to 0.05 mM. Cells were grown to about 1×10^6 cells/ml such that all cells were grown for three generations in the medium. Cells were then harvested by centrifugation at 3,000 × *g* for 5 min at 4°C and suspended in fresh TAP medium containing no phosphate at a cell density of 2×10^7 cells/ml. After the addition of ^{32}P-orthophosphate (ICN, in dilute HCl) at a concentration of 2 nM (175 kBq/ml), cells were incubated with vigorous shaking for 10 min or 20 min. To terminate the pulse, samples were transferred to a centrifuge tube containing 2 volumes of ice-cold TAP medium. Cells were pelleted by centrifugation at 3,000 × *g* for 3 min at 4°C, frozen in liquid nitrogen, and stored at -80°C prior to the RNA extraction. All steps were carried out within 5 min from the end of the pulse.

Hybridization analysis of ^{32}P-Labeled RNA

Plasmid DNAs, pUCGUS, pUCrbcL, pUCpsbA, pUCatpA, pUC16S, and pUC19 (1 μg/slot) were applied to Zeta-probe GT membrane (Bio-Rad) with Bio-Dot SF Microfiltration Apparatus (Bio-Rad), following the manufacture manual, and covalently linked to the membrane by exposure to UV light. Prehybridization was carried out for 1 hr and total radioactive RNA (10 μg) was applied onto the membrane and hybridization was performed for 72 hr at 65°C.

Table 2-2. Oligonucleotides used in this work.

oligo:	Sequence (5' to 3')
GUS5	CAGTCCCCCATGGTACGTCCTGTAGAA
GUS3	GCGGCATGCTTATTGTTTGCCTCCC
GUS3R	CCGCAGCAGGGAGGCAAACAATAA
RBCL5	GCACATCGATGGGTTTATAGGTATT
RBCL3	AACCATGGATATAAATAAATGTAAC
PSBA5	CGTCCTATATCGATACTCCGAAGGA
PSBA3	GCTGCCATGGGTTAATTTTTTTAAA
ATPA5	AATATCGATGACTTTATTAGAGGCAGTG
ATPA3	ATTGCCATGGAAAAGAAAAAATAAATAA
16S5	ATCCATGGAGAGTTTGATCCTGGCTC
16S3	CCTCTGTATTACCGCGGCTGCTGGCA
PF	TTATTTTAGGATCGTCAAAAGAAG
PR	ATGCTATTCACATAAACATCATG
PSBA30	CAACCGATGTATAAACGGTTTTCAG
ATPAC5	ATGGTAGATTTCGGTATCGTTTTCC
ATPAC3	AGCAGCTTTAGCTTGAGATTTAAATTC
PC6	TCCTTATTGAGCCTGTATTTGCTC
AAD3	GATCACTAAGGTAGTTGGCAAATAA

Results

The transformation of *C. reinhardtii* with *chimeric-uidA* genes

For the chloroplast transformation, *chimeric-uidA* expression vectors, pGrbcL, pGpsbA, and pGatpA were constructed (Fig. 2-3B). Each *chimeric-uidA* contains the *uidA* coding sequence, and the 3'-UTR of the *rbcL*. Each pGrbcL, pGpsbA, and pGatpA vector also consists of the putative promoter, and the full length 5'-UTR of the *rbcL*, *psbA*, or the *atpA*, respectively. *Chimeric-uidA*s were transcriptional and translational fusions, and flanked by fragments of the *rbcL* and *psaB* gene so as to introduce chimeric genes into the chloroplast genome by homologous recombination. Following by the particle bombardment of expression vectors to the *rbcL* disruption mutant DEVL, transformants were selected by photosynthetic competency for restoration of the *rbcL* (Fig. 2-1, also see Materials and Methods). The transformants, derived from the pGrbcL, pGpsbA, and the pGatpA were designated RG, PG, and AG, respectively. No difference was observed in the growth between the wild-type and all transformants.

The *Chlamydomonas* chloroplast contains approximately 70 to 80 genome copies. To confirm the homoplasmicity of the chloroplast genome in transformants, full length of the *rbcL*, *aadA*, and the *uidA* genes were amplified by PCR using a pair of primers PF/PR, AAD3/PC6, and GUS3R/PC6, respectively (Figs. 2-3 and 2-4). When the pair PF/PR was used, a 1.5 kb fragment corresponding to the *rbcL* was amplified in the wild-type and transformants except DEVL. In RG, additional 2.0 kb fragment was observed. As shown in Fig. 2-3, the PF/PR could anneal to the 5'- and 3'-UTR of the *chimeric-uidA* in RG. In DEVL, a 1.9 kb fragment corresponding to the *rbcL*, disrupted by the *aadA* cassette, was detected. When the pair AAD3/PC6 was used, no DNA fragments were amplified in all transformants. In addition, the transformants were sensitive to spectinomycin. These results indicated that the chloroplast genome of the transformants contained no *aadA* cassettes, and the genome was in the homoplasmic state.

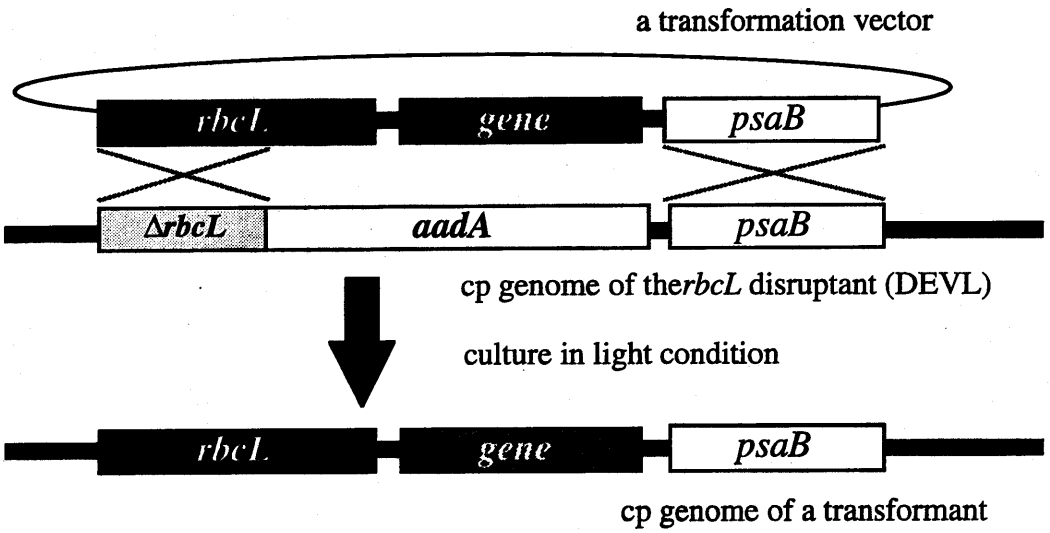

Figure 2-2. Homologous recombination of the chloroplast genome. Homologous recombination leads to the excision of the disrupted *rbcL* and the introduction of the full length *rbcL*, which complement photosynthesis for the transformant. The target gene is introduced between the *rbcL* and *psaB*.

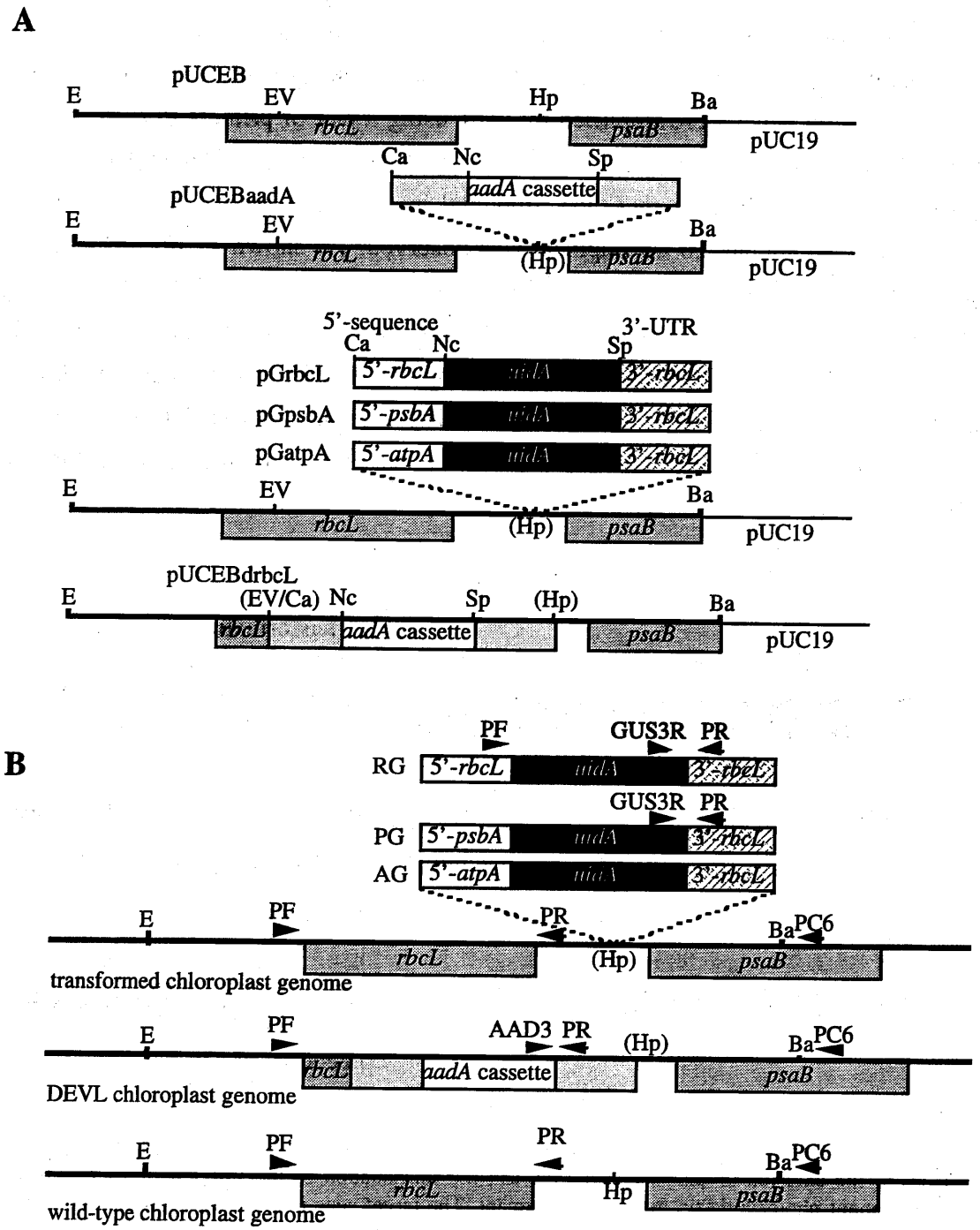

Figure 2-3. Plasmids for chloroplast transformation and genome in each transformant. (A) GUS expression plasmids, pGrbcL, pGpsbA, and pGatpA, and related plasmids, pUCEB and pUCEBaadA, are shown. The *uidA* coding sequence (filled boxes) was placed between the 5'-sequence of the chloroplast genes (open boxes) and the 3'-region of the *rbcL* gene (stippled boxes, *rbcL*). Each plasmid contains 5'-*rbcL* (0.37 kb), 5'-*psbA* (0.25 kb), and 5'-*atpA* (0.58 kb), respectively. The *chimeric-uidA* genes were cloned into a *Eco*RI-*Bam*HI chloroplast DNA fragment (thick line) between the *rbcL* and *psaB* genes (coding sequences are shown by shaded boxes). pUCEBdrbcL is the *rbcL*-deletion plasmid, in which the *rbcL* gene was replaced by an *aadA* cassette which consists of the *atpA* promoter, *aadA* coding sequence, and the *rbcL* 3'-UTR. Plasmid pUC19 is shown by a thin line. (B) PCR primers for confirmation of the homoplasmicity of transformants. Locations and directions of primers are indicated by arrowheads. E, *Eco*RI; EV, *Eco*RV; Ca, *Cla*I; Nc, *Nco*I; Sp, *Sph*I; Hp, *Hpa*I; Ba, *Bam*HI.

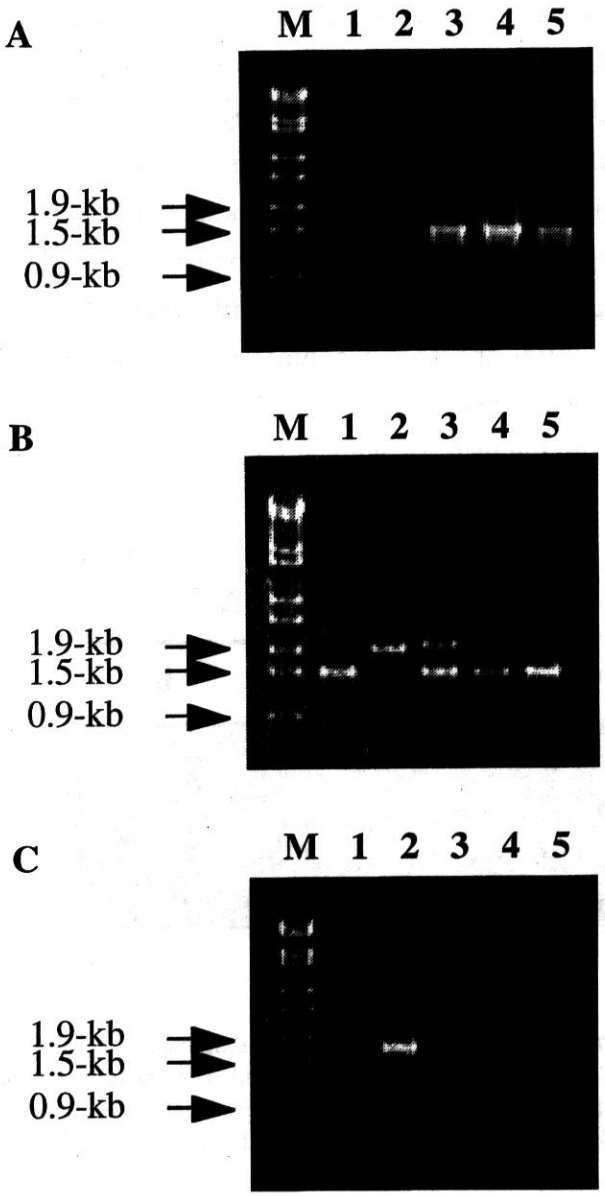

Figure 2-4. Confirmation of the chloroplast genome copies in each transformant. PCR fragments were analyzed by 1% agarose gel electrophoresis and detected by ethidium bromide staining. DNA fragments were amplified with primers GUS3R and PC6 for the *uidA* (A), primers PF and PR for the *rbcL* (B), and primers AAD3 and PC6 for the *aadA* (C). Lanes 1 to 5 were derived from the following cells; Lane 1, *C. reinhardtii* wild-type 137c; lane 2, DEVL; lane 3, RG; lane 4, PG; lane 5, AG. Lane M for λ/*Sty*I digest.

The accumulation of GUS protein in transformants

Because the *uidA* was transcriptionally and translationally fused to the promoter and 5'-UTR of the *rbcL*, *psbA*, or the *atpA*, transformants were thought to produce GUS fusion proteins. To examine the expression of the *chimeric-uidA* gene in each transformant, crude extracts were prepared from the mixotrophically grown cells. Then, proteins were separated by a 7.5% acrylamide SDS-PAGE, and GUS proteins were detected by western blotting with the anti-GUS antibody. Bands corresponding to the molecular size of the GUS protein (68 kDa) were observed in RG and AG, but not in PG (Fig. 2-5). Several non-specific signals were detected in both the wild-type and each transformant. GUS activities in crude extracts from photoautotrophically grown transformants were also quantified (Table 2-3). The enzyme activities in AG and RG were 130 ± 6.6 and 16 ± 5.5 nmol/min/mg protein, respectively. The activities in PG and the wild-type were less than 0.4 nmol/min/mg protein. The result of the enzyme activities was agreeable with the western analysis (Fig. 2-5).

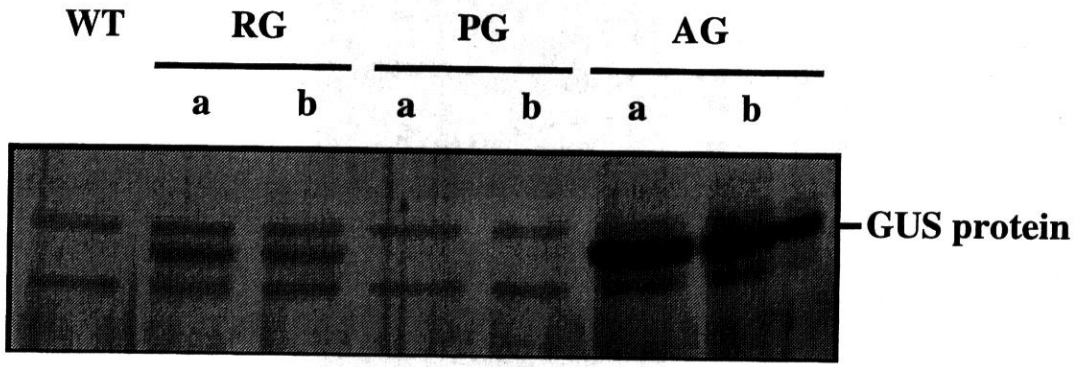

Figure 2-5. Western analysis of the crude extracts. Proteins (3 μg) extracted from cells grown in the TAP medium were separated by a 7.5% acrylamide SDS-PAGE, and GUS proteins were detected by western blotting. *C. reinhardtii* wild-type 137c (WT) and two independent clones (a and b) in each transformant (RG, PG, and AG) were analyzed. A signal 68 kDa in size corresponds to GUS protein.

The accumulation of *chimeric-uidA* transcripts in transformants

To distinguish the differential accumulation of *chimeric-uidA* mRNAs in each transformant, total RNA was extracted from photoautotrophically grown cells and analyzed by northern blotting (Fig. 2-6; Table 2-3). An approximately 2.0 kb transcript, an expected size from the *rbcL* transcription start site, was hybridized with the *uidA* probe in RG. Also, a 2.3 kb transcript, an expected size from the *atpA* transcription start site, was observed in AG. The *16S rRNA* probe was used for an internal control. The accumulation of the *chimeric-uidA* transcript in AG was 11-fold higher than the accumulation in RG. No transcript from the *chimeric-uidA*

was detected in PG. The result was consistent with the accumulation of the protein in each transformant.

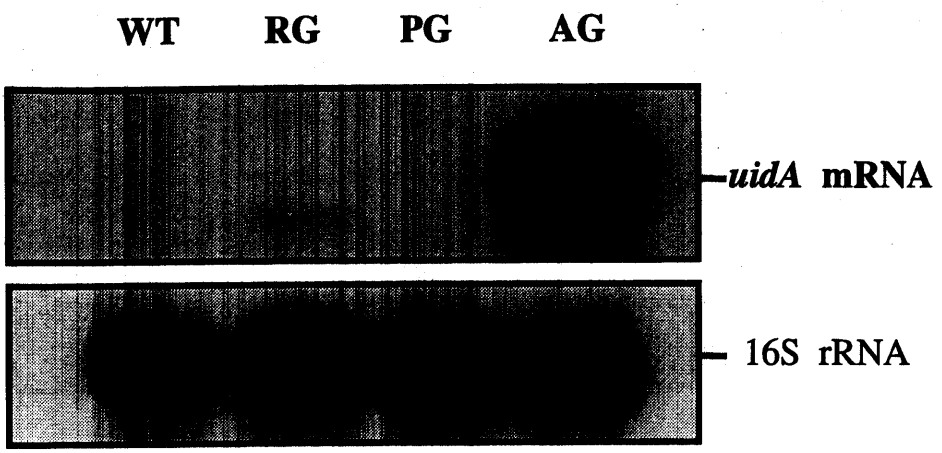

Figure 2-6. The accumulation of the *chimeric-uidA* mRNA in each transformant. Total RNA (5 μg) extracted from photoautotrophically grown cells was separated on a 1.5% formaldehyde/agarose gel, transferred to a nylon membrane, and hybridized with a 1.8 kb *Nco*I-*Sph*I fragment of the pT7GUS or a 0.5 kb *Eco*RI-*Hind*III fragment of the pUC16S as a probe for the *uidA* mRNA or 16S rRNA, respectively.

Transcription activities of *chimeric-uidA*s in transformants

To determine the relative transcription activity of the *chimeric-uidA*, transformants were subjected to *in vivo* pulse labeling (Fig. 2-7). The *chimeric-uidA* transcription activity in AG was 44-fold higher than the activity in RG. The activity in PG was 3.2-fold higher than the activity in RG. Transcription activity of the *chimeric-uidA* was almost comparable to the activity of endogenous *atpA* in AG. No significant changes were observed in transcription activities of endogenous genes among the transformants.

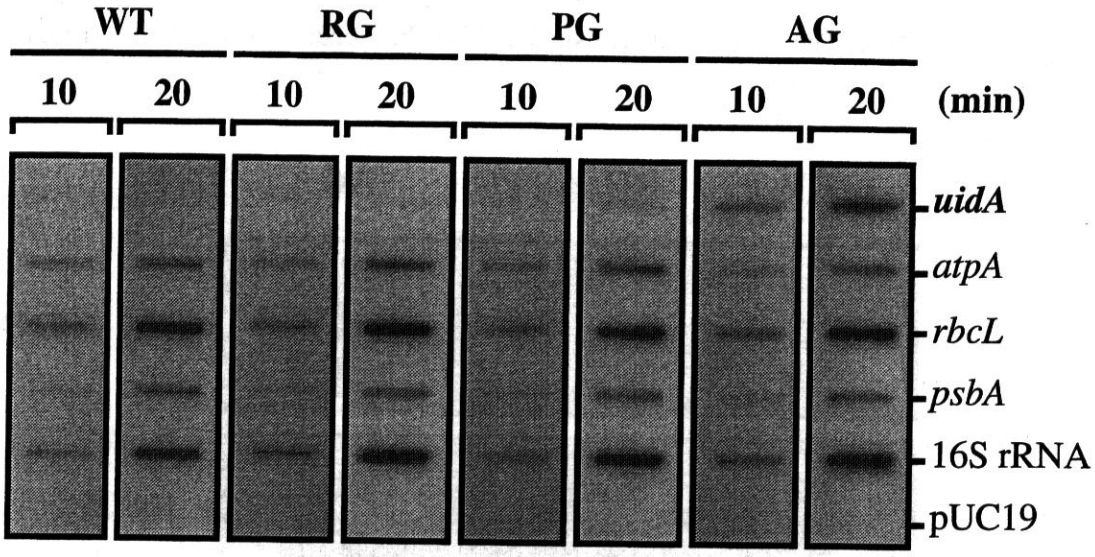

Figure 2-7. Transcriptional activity of the *chimeric-uidA* gene in each transformant. The wild-type (WT) and transformant (RG, PG, or AG) cells grown in the phosphate-depleted medium were labeled *in vivo* for 10 min or 20 min with ^{32}P-orthophosphate, and ^{32}P-labeled total RNA was hybridized to immobilized specific DNA. pUCGUS was a probe for the *chimeric-uidA* mRNA. pUCatpA, pUCrbcL, and pUCpsbA were for endogenous *atpA*, *rbcL*, and *psbA* mRNAs. pUC16S was for 16S rRNA as an internal control. Plasmid pUC19 was for a negative control.

Table 2-3. Gene expression of the *chimeric-uidA* in each transformant.

	WT	RG	PG	AG
GUS specific activity [a]	0.36 ± 0.0	16.5 ± 5.5	0.35 ± 0.1	130 ± 6.6
Relative activity of GUS protein [b]	0.02	1	0.02	7.88
Relative amount of *chimeric-uidA* mRNA [c]	N.D. [d]	1	N.D.	11
Relative transcription activity of *chimeric-uidA* [e]	-	1	3.16	44.1

		rbcL	*psbA*	*atpA*
Relative activity of endogenous gene [f]	-	1	0.423	0.058

a) Cells were grown in the M medium. Mean (± SD) of three independent experiments. b) Relative activity of GUS protein to the activity in RG. c) Relative amount of each *chimeric-uidA* mRNA to the amount in RG. d) N. D.; not detected. e) Relative transcription activity of each *chimeric-uidA* to the activity in RG. f) Relative transcription activity of endogenous genes to the *rbcL* (Table 2-1).

Discussion

Only the promoter and 5'-UTR are not sufficient for the full transcription activity.

The *chimeric-uidA*s, containing putative promoters and 5'-UTRs of the *rbcL*, *psbA*, and the *atpA*, did not reflect the endogenous gene expression, especially in the relative activity of transcription. In transformants, the *atpA* 5'-sequence promoted the highest transcription activity for the *chimeric-uidA*, followed by the *psbA* sequence, then the *rbcL* sequence (Fig. 2-7, Table 2-3). However, the *rbcL* is most actively transcribed except the*16S rRNA* among endogenous genes (Table 2-1). In RG and PG, transcription activities of the *chimeric-uidA* to activities of endogenous genes were drastically reduced. The results implicate that the element other than the promoter and 5'-UTR, probably a part of the coding sequence, enhances the transcription activity of the *rbcL* or *psbA*. A 58 bp fragment of the *rbcL*, well within the coding sequence, appeared to be essential for the transcription activity of the endogenous *rbcL* (Uwe Klein, a personal communication). Such transcription enhancers in the *rbcL* and *psbA* may allow the cell to accumulate the largest mRNA pools of both genes. Compared to my results with *C. reinhardtii*, the *rbcL* core promoter is sufficient to obtain the wild-type transcription activity in tobacco (Shiina et al., 1998). The transcription machinery in *C. reinhardtii* might be different to the machinery in higher plants. On the other hand, the difference in transcription between the *chimeric-uidA* in AG and endogenous *atpA* gene was not significant. In case of the *atpA*, the transcription activity may simply require the promoter. Still, the result provides another speculation. While all promoter sequences in the *atpA*, *rbcL*, and the *psbA* have common prokaryotic features, the 5'-UTR of the *atpA* differs in size. The *atpA* 5'-UTR contains 424 bp, while the *rbcL* 5'-UTR is 92 bp and *psbA* 5'-UTR is 90 bp. Therefore, the *atpA* 5'-UTR may contain an enhancer sequence for transcription. The deletion analysis of the 5'-UTR would confirm this hypothesis.

The coding sequence affects the mRNA stability.

In this study, transcription activities did not always reflect the accumulations of the *chimeric-uidA* transcript. In PG, the transcription activity of the *psbA-uidA* was approximately 3-fold higher than the activity of the *rbcL-uidA* in RG (Fig. 2-7 and Table 2-3). In RG, the accumulation of the *chimeric-uidA* transcript was well observed. Nevertheless, no accumulation of the *chimeric-uidA* transcript was detected in PG (Fig. 2-6). The results indicate that the 5'-UTR is not sufficient for the mRNA stability in the *psbA-uidA*. Along with the enhancement of the transcription activity, the coding sequence of the *psbA* may stabilize its transcript.

CHAPTER III

Effects of the coding sequence on the chloroplast gene expression

Introduction

In Chapter II, relative transcription activities of *chimeric-uidA*s in RG and PG were drastically reduced from activities of endogenous genes. The result suggests that only the promoter and 5'-UTR are not sufficient for transcription activity of the chloroplast gene. Moreover, the transcription activity of the *chimeric-uidA* was well observed, but the mRNA accumulation was not detected in PG, suggesting that only the promoter and 5'-UTR are not sufficient for mRNA stability as well as for transcription activity. These results imply that presumably the coding sequence, in addition to the promoter and 5'-UTR, might be required for the chloroplast gene expression. In fact, the fusion of the *rbcL* coding sequence with the *uidA* gene was transcribed at the same rate as the endogenous *rbcL* gene (Klein et al., 1994). However, the *chimeric-uidA* was transcriptional but non-translational fusion in this experiment. The *uidA* translational fusion with the *petD* sequence predicted that translation affects the mRNA accumulation, presumably through changing its stability (Sakamoto et al., 1993). Therefore, it is possible that the translational fusion shows different result with the non-translational fusion in the gene expression.

In this chapter, I examined the effects of the coding sequence on the gene expression, and confirmed the influence of the *rbcL* coding sequence with the *chimeric-uidA* translational fusion gene on the expression. First, the transformant harboring the *uidA* fused to the sequences from the *rbcL*, *psbA*, or the *atpA* was obtained. Each sequence includes the promoter, 5'-UTR, and the portion of the coding sequence from the endogenous gene. Then, the *chimeric-uidA* gene expression in the transformant was observed at the level of transcription, post-transcription, and translation. The analysis showed that the coding sequences of the *rbcL* and *psbA*, but not of the *atpA*, enhanced promoter activities. The result also suggested that the coding sequence of the *psbA* increases mRNA stability. Moreover, two *atpA*-derived transformants showed that the accumulation of the GUS protein contradicted to the accumulation of the transcript.

Materials and Methods

Strain and culture conditions

C. reinhardtii wild-type 137c and transformants were grown in TAP or M medium in the light or in the dark at 28°C. Where necessary, the medium were solidified with 2% agar and

supplemented with spectinomycin. Cells grown to 2×10^6 cells/ml in a liquid medium were harvested for all RNA and protein isolations except for the *in vivo* RNA labeling study.

DNA constructs

Recombinant DNA plasmids were prepared by conventional procedures. Bacterial host was *E. coli* DH5α.

For PCR amplifications of the promoter, 5'-UTR, and N-terminal portion of the *rbcL*, whole genome was isolated from *C. reinhardtii* wild-type 137c for a template. PCR primer pairs were RBCL5 and RBCL112 for a 402 bp fragment containing a sequence which encodes N-terminal 7 amino acids, RBCL5 and RBCL124 for a 414 bp fragment containing a sequence which encodes N-terminal 11 amino acids, and RBCL5 and RBCL172 for a 462 bp fragment containing a sequence which encodes N-terminal 27 amino acids (Table 3-1). All fragments contain the *rbcL* promoter and 5'-UTR. These fragments were subcloned into pT7Blue T-Vector, and excised with *Cla*I and *Nco*I. The *Cla*I-*Nco*I fragments were introduced into *Cla*I-*Nco*I digested pUCEBGUS. Resulting plasmids were designated pRGF7, pRGF11, and pRGF27, respectively. As well as the fragment of *rbcL*, a fragment containing the *psbA* promoter, 5'-UTR, and sequence which encodes N-terminal 29 amino acids of D1 protein was amplified with a primer pairs PSBA5 and PSBA29 (Table 3-1). A 0.3 kb fragment was subcloned into pT7Blue T-Vector, and excised with *Cla*I and *Nco*I. The *Cla*I-*Nco*I fragment was introduced into *Cla*I-*Nco*I digested pUCEBGUS, resulting the plasmid pPGF29.

For an *atpA* derivative, a 1.8 kb *Nco*I-*Sph*I fragment of pT7GUS (Chapter II) was introduced into *Nco*I-*Sph*I digested pUCEBaadA (Chapter II). The resulting plasmid was designated pAGF25 in which the *atpA* sequence encodes N-terminal 25 amino acids of α subunit of ATP synthase was fused with the *uidA* gene.

Assay of the *chimeric-uidA* gene expression

Individual steps of the procedure were done as described in Chapter II.

Table 3-1. Oligonucleotides used in this work.

oligo:	Sequence (5' to 3')
RBCL5	GCACATCGATGGGTTTATAGGTATT
RBCL112	TGCCATGGTTTCTGTTTGTGGAACCAT
RBCL124	GACCATGGCACCTGCTTTAGTTTCTGT
RBCL172	GTCCATGGGTGTGTAGTATGTTAAACG
PSBA5	CGTCCTATATCGATACTCCGAAGGA
PSBA29	GACTTTTGGCAAATAGGTACCCAAC

Results

Transformants harboring the portions of the *rbcL* coding sequence

To investigate the effect of the *rbcL* coding sequence on the *chimeric-uidA* gene expression, transformants RG, RGF7, RGF11, and RGF27 were obtained (Fig. 3-1). All transformants have *rbcL-uidA* transcriptional and translational fusion genes. RG harbors the *chimeric-uidA* in which the *uidA* was fused to only the promoter and 5'-UTR from the *rbcL* gene (also see Chapter II). RGF7, RGF11 or RGF27 carries the *chimeric-uidA* consists of the 5'-sequence and a portion of the *rbcL* coding sequence. Each fragment of the *rbcL* coding sequence corresponds to the N-terminal 7, 11, or 27 amino acid residues of the RuBisCO large subunit for RGF7, RGF11, or RGF27, respectively.

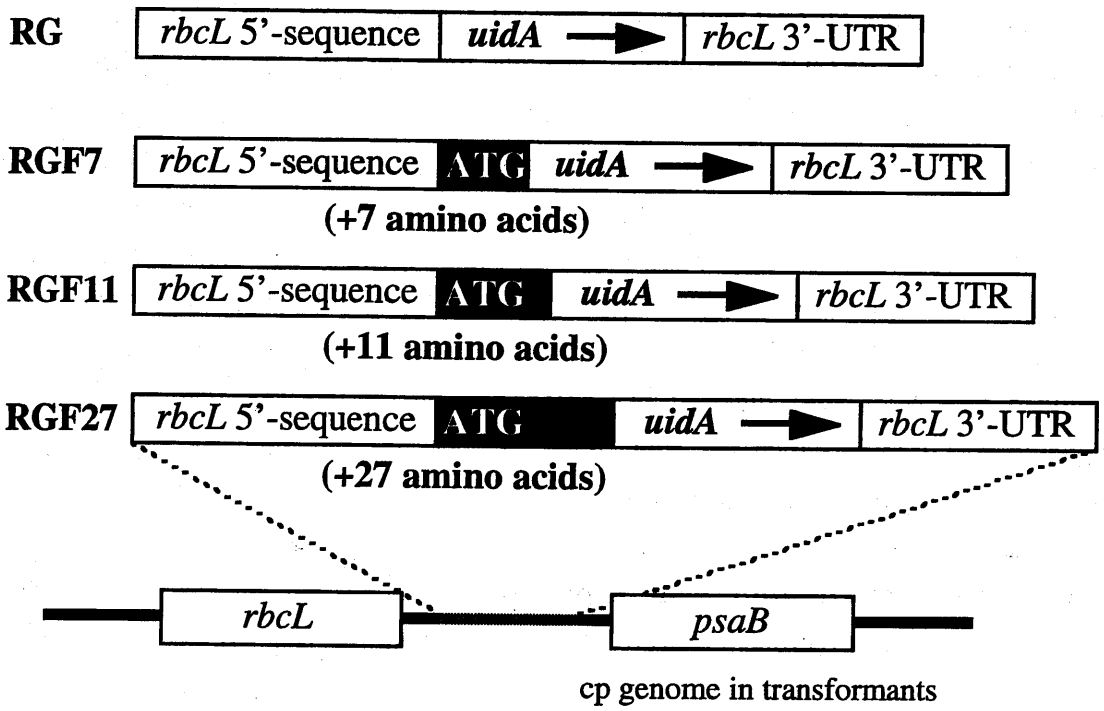

Figure 3-1. All transformants have *rbcL-uidA* transcriptional and translational fusion genes. RG harbors the *chimeric-uidA* in which the *uidA* was fused to only the promoter and 5'-UTR (5'-sequence) from the *rbcL* gene (see Chapter II). RGF7, RGF11 or RGF27 carries the *chimeric-uidA* consists of the promoter, 5'-UTR, and a portion of the *rbcL* coding sequence. Each fragment of the *rbcL* coding sequence corresponds to the N-terminal 7, 11, or 27 amino acid residues of the RuBisCO large subunit for RGF7, RGF11, or RGF27, respectively.

Gene expression of the *rbcL-uidA* in transformants

Transcription activities in RG, RGF7, RGF11, and RGF27 were determined by *in vivo* pulse labeling (Fig. 3-2 and Table 3-2). Relative transcription activity of the *chimeric-uidA* to the endogenous *rbcL* were 16.7%, 17.5%, 28.2% for RG, RGF7, and RGF11, respectively. The *chimeric-uidA* transcription activity in RGF27 was restored to 75.5% of the activity of endogenous *rbcL* gene. No significant changes were observed in transcription activities of endogenous *rbcL* among the transformants.

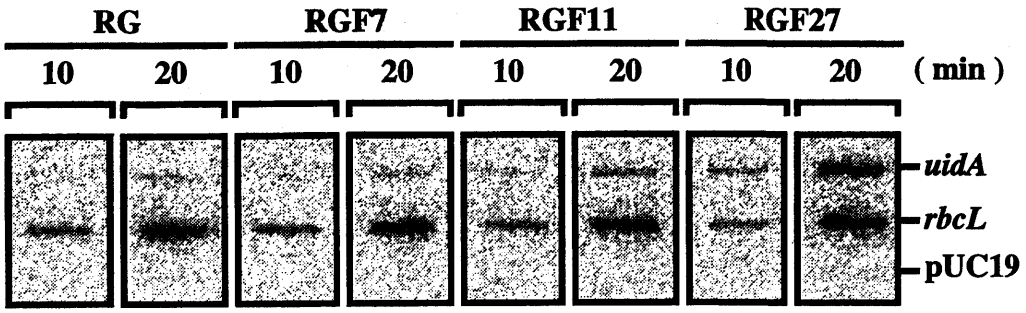

Figure 3-2. Transcription activity of the *chimeric-uidA* gene in each transformant. Transformants (RG, RGF7, RGF11, and RGF27) grown in the phosphate-depleted medium were labeled *in vivo* for 10 or 20 min with ^{32}P-orthophosphate, and ^{32}P-labeled total RNA was hybridized to immobilized specific DNA. pUCGUS was a probe for the *chimeric-uidA* mRNA, pUCrbcL was for endogenous *rbcL* mRNA, and plasmid pUC19 was for a negative control.

Table 3-2. Relative transcription activity of the *chimeric-uidA* gene in each transformant.

	RG	RGF7	RGF11	RGF27
relative transcription activity [a] (%)	16.7	17.5	28.2	75.5

a) Relative *chimeric-uidA* transcription activity in each transformant to the activity of endogenous *rbcL*.

In northern analysis, differential accumulations of *chimeric-uidA* transcripts among transformants were observed (Fig. 3-3). Transcripts from each chimeric gene were approximately 2.0 kb in size but each showed a small difference, corresponding the differential length of the fusion of the *rbcL* coding sequence. The transcript was most accumulated in RGF27 which harbors the longest fragment of the *rbcL* coding sequence. Accumulations in RG, RGF7, and RGF11 were 12.3%, 17.3%, and 41.5% relative to the accumulation in the

RGF27 (Table 3-3). The mRNA accumulations was closely correlated to the relative transcription activities in individual transformants.

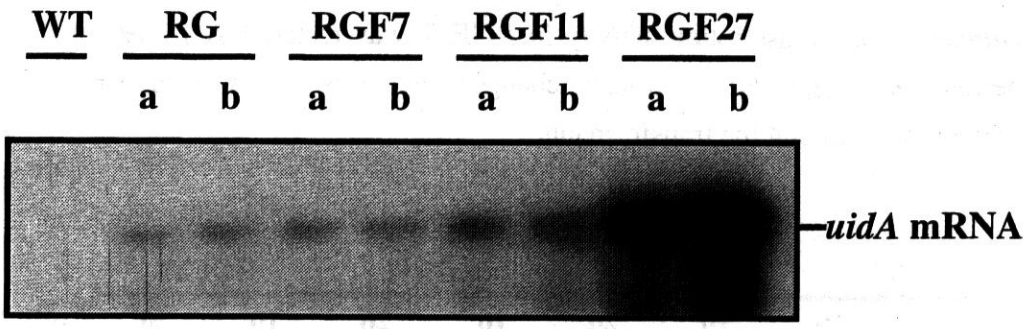

Figure 3-3. The accumulation of the *rbcL-uidA* mRNA in each transformant. Total RNA (5 μg) extracted from TAP-grown cells was separated on 1.5% formaldehyde/agarose gel, transferred to nylon membrane, and hybridized with a 1.8 kb *Nco*I-*Sph*I fragment of the pT7GUS. *C. reinhardtii* wild-type 137c and two independent clones (a, b) of each transformant were analyzed.

Table 3-3. Relative amount of *chimeric-uidA* mRNA accumulation in each transformant.

	RG	RGF7	RGF11	RGF27
relative mRNA amount[a)] (%)	12.3	17.3	41.5	100

a) Relative *uidA* mRNA amount in each transformant to the amount in RGF27.

The accumulations of the proteins were analyzed by western blotting since the fusion proteins had no GUS activity (Fig. 3-4). Bands corresponding to the molecular size of the GUS protein (68 kDa) were observed in all transformants, but each band showed a small difference in size, consistent with the different size of the fusion protein. Considering the size of each protein, lower bands observed in RGF7, RGF11, and RGF27 were non specific proteins. As is the case for the transcript, the GUS protein was most accumulated in RGF27. The accumulations in RG, RGF7, and RGF11 were 16.2%, 20.8%, and 29.5% relative to the accumulation in RGF27 (Table 3-4).

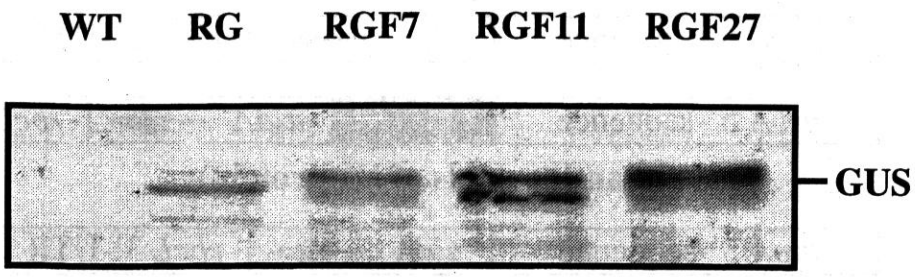

Figure 3-4. Western analysis of crude extracts. Proteins (3 μg) extracted from TAP-grown cells were separated by a 7.5% acrylamide SDS-PAGE, and GUS proteins were detected by western blotting.

Table 3-4. Relative amount of GUS protein each transformant.

	RG	RGF7	RGF11	RGF27
relative amount of GUS protein[a] (%)	16.2	20.8	29.5	100

a) Relative amount of GUS protein in the transformant to the amount in RGF27.

Transformants harboring a portion of the *psbA* or *atpA* coding sequence

To examine the effects of coding sequences of the *psbA* and *atpA*, as well as the *rbcL*, transformants PG and PGF29 for the *psbA*, or AG and AGF25 for the *atpA* were obtained (Fig. 3-5, also see Chapter II). PG or AG harbors the *chimeric-uidA* in which the *uidA* was fused only to the promoter and 5'-UTR of the *psbA* or *atpA*, respectively. In PGF29, the promoter, 5'-UTR, and a portion of the *psbA* coding sequence which corresponds to the N-terminal 29 amino acid residues of the D1 protein, were fused with the *uidA*. In AGF25, the *chimeric-uidA* contains a portion of the *atpA* coding sequence, corresponding to the N-terminal 25 amino acid residues of ATP synthase α subunit. These *chimeric-uidA*s are the transcriptional and translational fusion genes, as is the case for the *rbcL-uidA*.

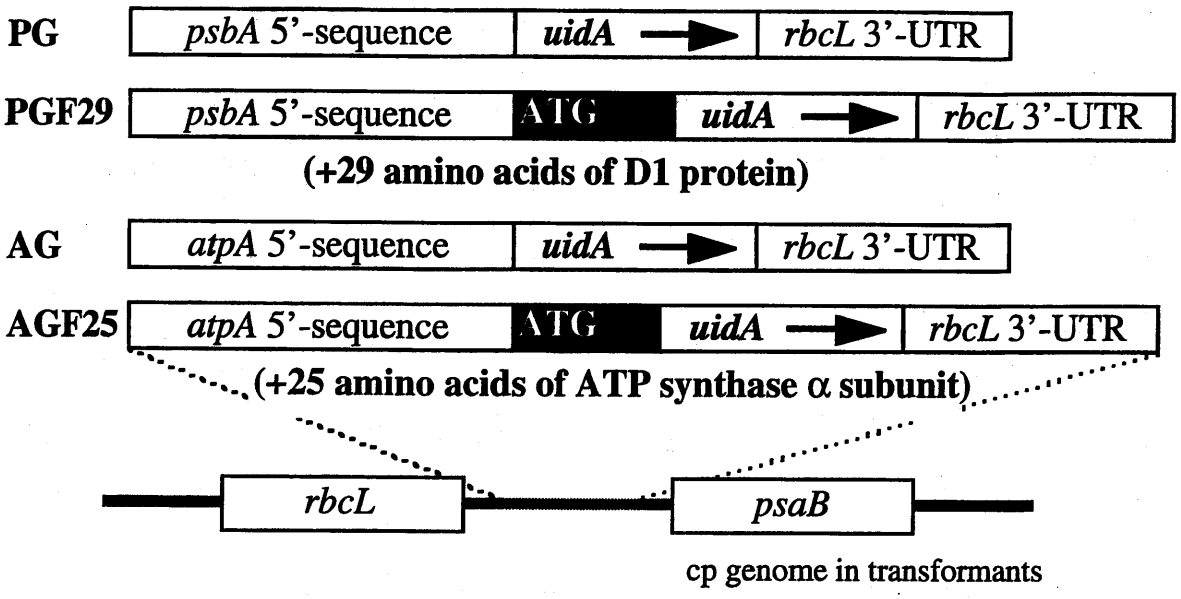

Figure 3-5. Transformants harboring transcriptional and translational fusion of the *psbA-uidA* or *atpA-uidA* genes. PG or AG carries the *chimeric-uidA* in which the *uidA* was fused to only the promoter and 5'-UTR (5'-sequence) from the *psbA* or *atpA* gene, respectively (see Chapter II). PGF29 carries the *chimeric-uidA* containing of the promoter, 5'-UTR, and a portion of the *psbA* coding sequence which corresponds to the N-terminal 29 amino acid residues of D1 protein. AGF25 carries the *chimeric-uidA* containing of the promoter, 5'-UTR, and a portion of the *atpA* coding sequence which corresponds to the N-terminal 25 amino acid residues of ATP synthase α subunit.

Gene expression of the *psbA-uidA* in transformants

Transcription activity of the *psbA-uidA* was observed in PG, and 8.3% of the activity of endogenous *psbA* gene (Fig. 3-6, Table 3-5). In PGF29, the transcription activity of the *psbA-uidA* was restored to 70.8% of the endogenous *psbA* transcription activity. Small differences were observed in signal intensities of internal controls with the *atpA* and *psbA* probes between PGF29 and PG. Nevertheless, relative transcription activities of the endogenous *psbA* and *atpA* gene had no change between transformants. Therefore, I concluded that total RNA amounts applied to the hybridization were different. The result showed that the coding sequence of the *psbA*, as well as the *rbcL*, enhanced transcription activity.

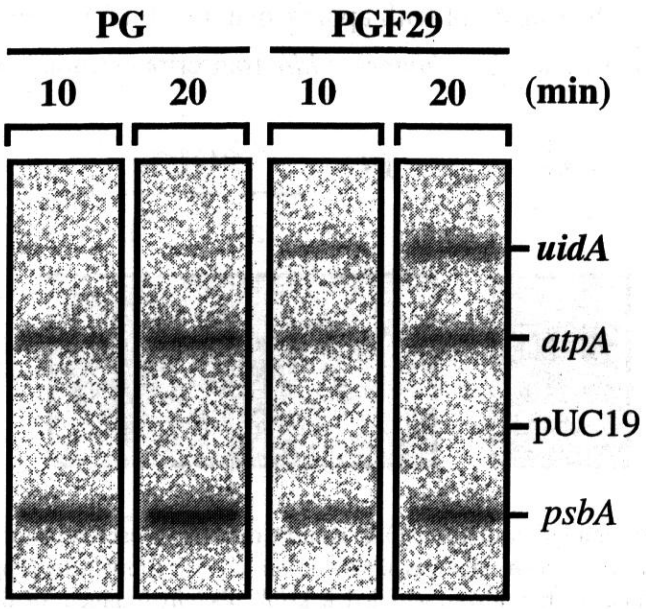

Figure 3-6. Transcription activity of the *chimeric-uidA* gene in each transformant. Transformants (PG and PGF29) grown in the phosphate-depleted medium were labeled *in vivo* for 10 or 20 min with ^{32}P-orthophosphate, and ^{32}P-labeled total RNA was hybridized to immobilized specific DNA. pUCGUS was a probe for the *chimeric-uidA* mRNA, pUCatpA and pUCpsbA were for mRNAs from endogenous *atpA* and *psbA*, respectively. Plasmid pUC19 was a probe for a negative control.

Table 3-5. Relative activity of the *chimeric-uidA* in each transformant.

	PG	PGF29
relative transcription activity [a] (%)	8.3	70.8

a) Relative *chimeric-uidA* transcription activity in the transformant to the activity of endogenous *psbA*.

In northern analysis, the transcript with approximately 2.0 kb in size was observed in PGF29 (Fig. 3-7). On the other hand, the *chimeric-uidA* transcript accumulation was not detected in PG.

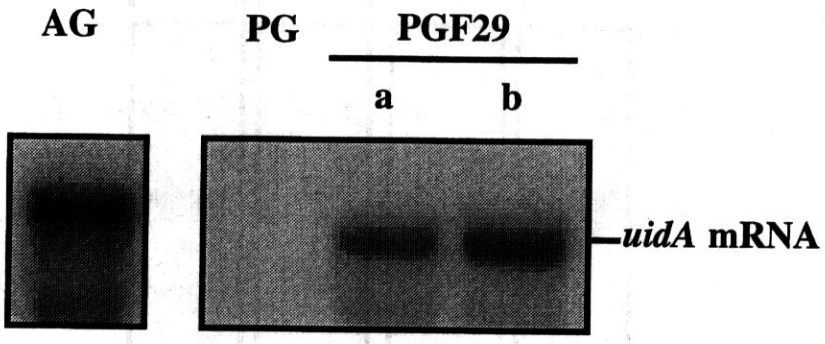

Figure 3-7. The accumulation of the *chimeric-uidA* mRNA in each transformant. Total RNA (5 μg) extracted from TAP-grown cells was separated on a 1.5% formaldehyde/agarose gel, transferred to a nylon membrane, and hybridized with a 1.8 kb *Nco*I-*Sph*I fragment of the pT7GUS. PG, and two independent clones of PGF (a, b) were analyzed. AG was compared as a positive control.

GUS specific activities in PGF29 was 40.2 ± 12.2 nmol/min/mg protein, whereas no activity was detected in PG (Table 3-6).

Table 3-6. GUS specific activity in each transformant.

	PG	PGF29
GUS specific activity [a] (nmole/min/mg protein)	N.D. [b]	40.2 ± 12.2

a) Cells were grown in TAP medium. b) N. D.; not detected. Mean (± SD) of three independent experiments.

Gene expression of the *atpA-uidA* in transformants

Transcription activities of the *chimeric-uidA*s were enhanced by the portion of coding sequences from the *rbcL* and *psbA*. On the contrary, the transcription activity in AG was almost comparable, or even appeared to be higher than the activity in AGF25, which harbors the portion of the *atpA* coding sequence (Fig. 3-8). On the other hand, the steady-state level of the mRNA in AGF25 was approximately 4-fold lower than in AG (Fig. 3-9 and Fig. 3-11B).

GUS specific activity in AGF25 was 480 ± 19.9 nmol/min/mg protein, approximately 3.7-fold higher than the activity in AG; 130 ± 6.6 nmol/min/mg protein (Fig. 3-11A). The result contradicted the differential accumulation of the mRNA between transformants. The difference in the enzyme activities was consistent with the result of the western analysis (Fig. 3-10),

showing that the differential enzyme specific activity was caused by the differential protein accumulation.

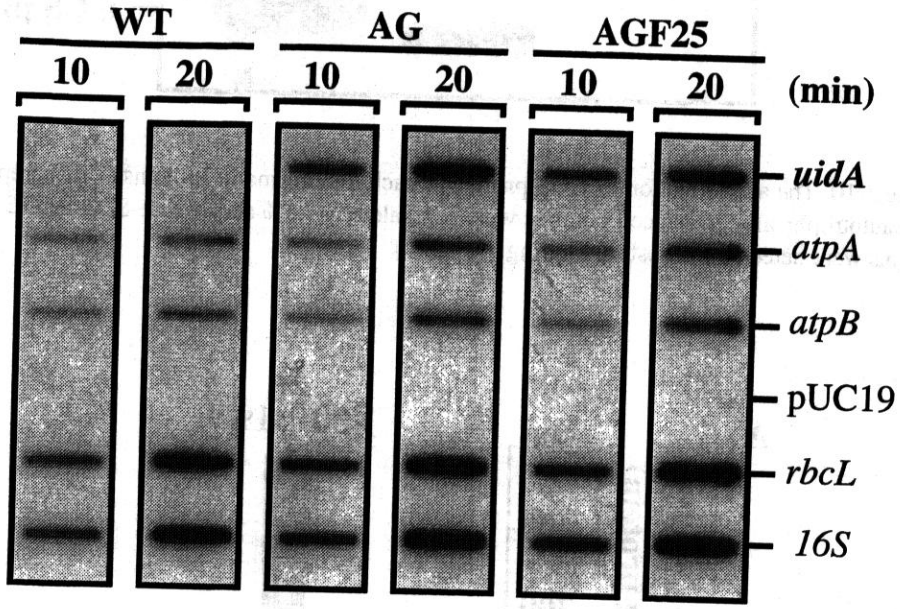

Figure 3-8. Transcription activity of the *chimeric-uidA* in each transformant. *C. reinhardtii* wild-type 137c (WT) and transformant (AG or AGF25) grown in the phosphate-depleted medium were labeled *in vivo* for 10 or 20 min with ^{32}P-orthophosphate, and ^{32}P-labeled total RNA was hybridized to immobilized specific DNA. pUCGUS was a probe for the *chimeric-uidA* mRNA, pUCatpA and pUCpsbA were for mRNAs from endogenous *atpA* and *psbA*, respectively. Plasmid pUC19 was for a negative control.

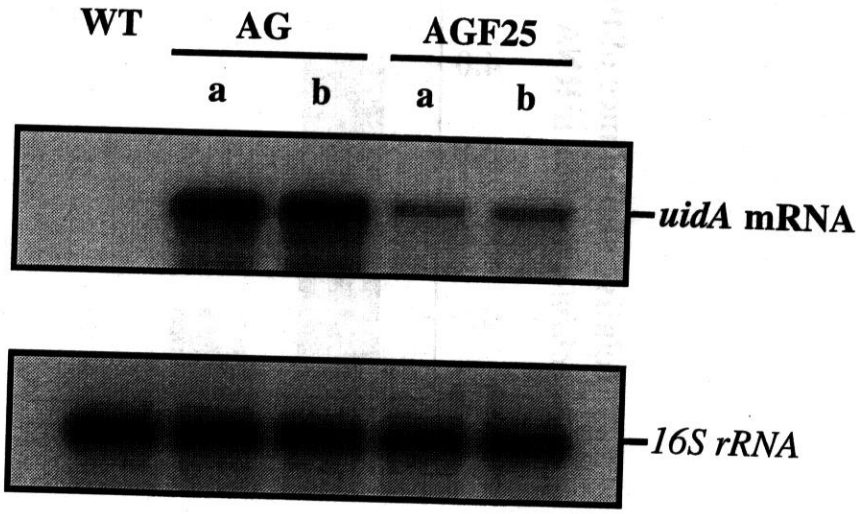

Figure 3-9. The accumulation of the *atpA-uidA* mRNA in each transformant. Total RNA (5 μg) extracted from TAP grown cells was separated on a 1.5% formaldehyde/agarose gel, transferred to a nylon membrane, and hybridized with a 1.8 kb *Nco*I-*Sph*I fragment of the pT7GUS. *C. reinhardtii* wild-type 137c (WT) and two independent clones of transformants AG and AGF25 were analyzed. A 0.5 kb *Eco*RI-*Hin*dIII fragment of the pUC16S was a probe for the internal control.

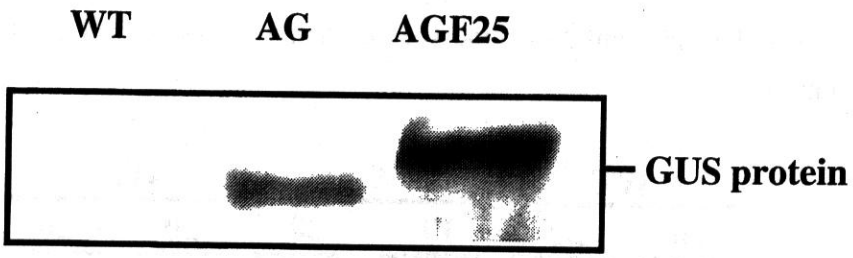

Figure 3-10. The accumulation of GUS protein in each transformant. Proteins (1 μg) prepared from photoautotrophically grown cell cultures were separated by 7.5% acrylamide SDS-PAGE, and GUS proteins were detected by western blotting.

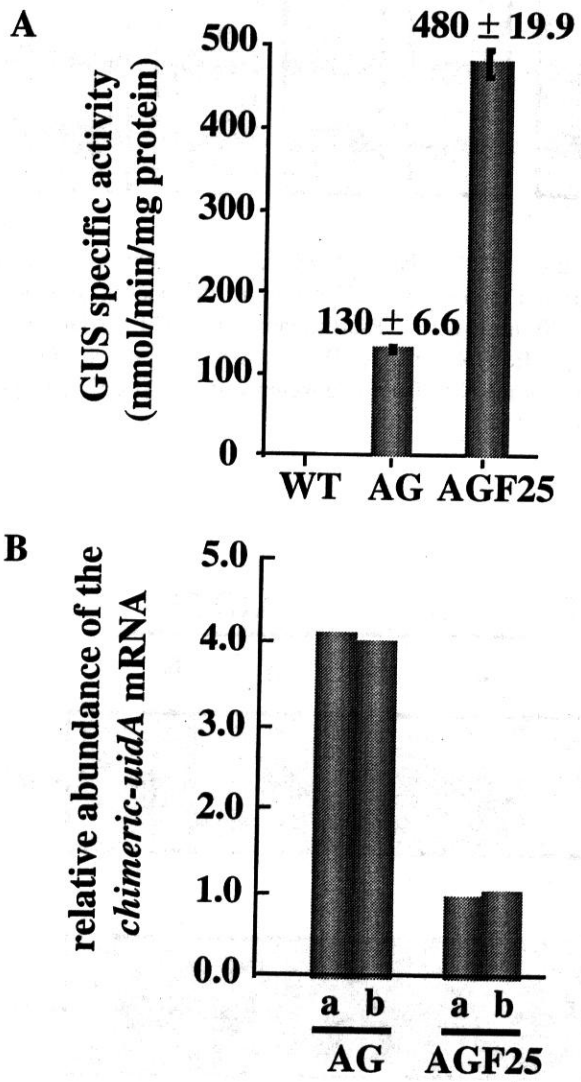

Figure 3-11. Comparison of GUS specific activities and amounts of the transcript between AG and AGF25. (A) GUS specific activity in transformants. Crude extracts were prepared from photoautotrophically grown cell cultures. Data are the means (± SD) of three independent experiments. (B) Quantitative analysis of the transcripts accumulation from northern blotting.

Discussion

Coding sequences of the *rbcL* and *psbA* enhance the promoter activity.

The transcription activity of the *chimeric-uidA*, was 75.5% of the activity of endogenous *rbcL* gene in RGF27. On the other hand, the activity was only 16.7%, 17.5%, and 28.2% of the transcription activity of the endogenous *rbcL* in RG, RGF7 and RGF11, respectively (Fig. 3-2 and Table 3-2). The results suggest that the *rbcL* coding sequence enhances the activity of the *rbcL* promoter, and the portion of the coding sequence which corresponds to the N-terminal 27 amino acid residues is almost sufficient for this enhancement. The accumulations of the *chimeric-uidA* mRNA and GUS protein were comparable to those of the transcription activities in all the *rbcL-uidA* derived transformants. The result of transcription activities in these transformants were consistent with the previous observation that the *rbcL* coding sequence was supposed to be essential for the full activity of the *rbcL* promoter, although untranslational gene had been used in the experiment (Klein et al., 1994). Now the enhancing fragment is determined as a 58 bp stretch (Uwe Klein, a personal communication).

In the chloroplast genes, not only the *rbcL* but also the *psbA* coding sequence enhanced the promoter activity (Fig. 3-6 and Table 3-5). Transcription activity of the *chimeric-uidA* observed in PG was 8.3% relative to the activity of endogenous *psbA* gene. In PGF29, transcription activity of the *chimeric-uidA* was restored to 70.8% of the endogenous *psbA* transcription activity. The result suggests that the portion of the coding sequence which corresponds to the N-terminal 29 amino acid residues is indispensable for the full promoter activity in the *psbA*. On the other hand, in AG, the transcription activity of the *chimeric-uidA* which does not contain the *atpA* coding sequence exhibited almost comparable to the endogenous *atpA* gene (Fig. 3-8). The activity in AGF25 had no significant difference to the activity in AG. In case of the *atpA*, the coding sequence did not enhance the promoter activity and appears to be not essential for transcription. As described in Chapter II, it is possible that the 5'-UTR of the *atpA* enhances the basal promoter activity. Differential effects of the *rbcL*, *psbA*, and the *atpA* coding sequences on transcription suggest that the differential transcription regulations exist in the chloroplast genome.

The mechanism of the enhancement of the promoter activity is not known, though I presume following models. The coding sequence may change the conformation of the promoter region and enhance the formation of transcription initiation complex (Fig. 3-12A). Another possibility is that the coding sequence may enhance the elongation of transcription (Fig. 3-12B). The conformation change or the enhancement of the elongation may be promoted by the coding sequence itself or the some factor which binds to the coding sequence. A mutation in the *C. reinhardtii* nuclear genome reduced the transcription activity of the *rbcL* gene (Hong and Spreitzer, 1998). It is possible that the mutation is mapped on the gene for the factor, which binds to the *rbcL* coding sequence and enhances the promoter activity.

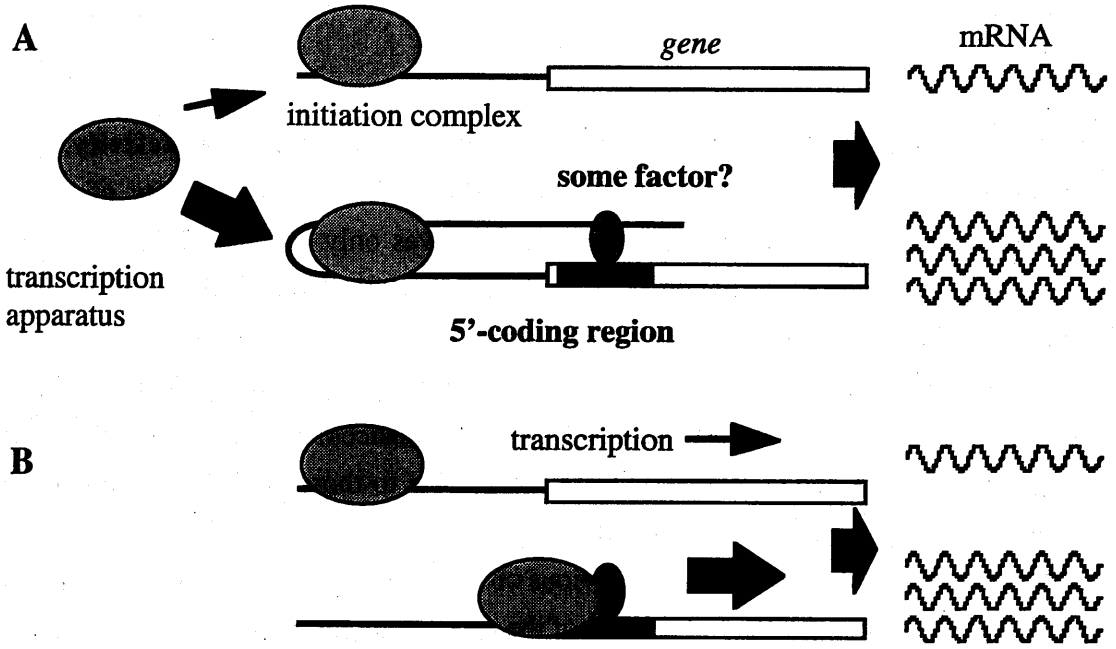

Figure 3-12. Hypothetical mechanisms for the enhancement of transcription. (A) The coding sequence may change the conformation of the promoter region and enhance the formation of transcription initiation complex. (B) The coding sequence may enhance the elongation of transcription. This conformation change or the enhancement of the elongation may be promoted by the coding sequence itself or the some factor which binds to the coding sequence.

The *psbA* coding sequence affects mRNA stability.

In PG, the transcription activity of the *chimeric-uidA* was observed. However no accumulation of the *chimeric-uidA* transcript was detected (Figs. 3-6 and 3-7, also see discussion of Chapter II). On the other hand, PGF29 showed the higher *chimeric-uidA* transcription activity than in PG, and indeed, the accumulation of the *chimeric-uidA* transcript was observed (Fig. 3-7). The result suggests that the *psbA* coding sequence enhances both transcription and the mRNA stability. The portion of the *psbA* coding sequence may change the secondary structure of the transcript and prevent the transcript from degradation (Fig. 3-13). Or, the RNA-binding protein which binds to the portion of the *psbA* coding sequence may stabilize the transcript, as is the role of the 5'-UTR.

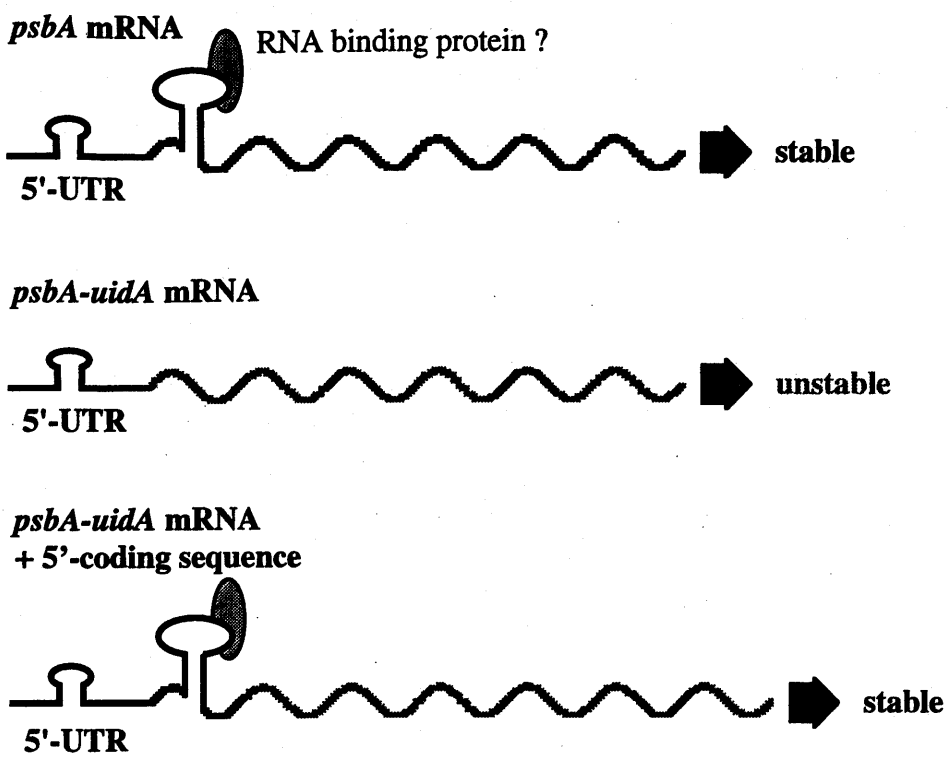

Figure 3-13. Hypothetical effect of the *psbA* coding sequence on the *chimeric-uidA* mRNA stability. The *psbA* coding sequence may enhance both transcription and mRNA stability. The portion of the *psbA* coding sequence may change the secondary structure of the transcript and protect the transcript from degradation. Or, the RNA-binding protein which binds to the portion of the *psbA* coding sequence may stabilize the transcript.

Although I could not estimate the effect of the *rbcL* coding sequence on mRNA stability in my experiments, previous report suggested that the portion of the *rbcL* coding sequence also stabilize the mRNA (Salvador et al., 1993). The stabilizing effect of the *rbcL* and *psbA* coding sequences, along with the role of transcription enhancement, may allow the cell to obtain the two largest mRNA pools in the chloroplast.

Incoordination between accumulations of the transcript and protein in the *atpA-uidA*-harboring transformants.

Comparing with the result of the *rbcL*- and *psbA-uidA* derivatives, transformants AGF25 and AG were exceptional in the accumulations of the transcript and the protein. In AGF25 and AG, transcription activities showed only a small difference (Fig. 3-8). However, the accumulation of the *chimeric-uidA* mRNA in AGF25 was approximately 4-fold lower than in AG (Fig. 3-11B). These results suggest that mRNA stabilities in the two transformants are different. The weaker signal in AGF25 than the signal in AG observed in the *chimeric-uidA* transcription activity assay may have come from the shorter half-life of the transcript in AGF25. On the other hand, GUS protein accumulation in AGF25 was approximately 4-fold higher than in AG (Fig. 3-

11A). Two explanations for the reason of these differences are conceivable. One is that a portion of the *atpA* coding sequence may have changed the secondary structure of the *chimeric-uidA* mRNA that would influence the mRNA stability, and/or the corresponding peptide of ATP synthase α subunit would have affected the stability of the fusion protein. Another possibility is that the translation efficiencies for the *chimeric-uidA* between AGF25 and AG are different, and that brought differential GUS protein accumulations. In addition to the effect on the protein accumulations, differential translation efficiencies may have changed the mRNA stabilities in both transformants, resulted in differential mRNA accumulations. A portion of the *atpA* coding sequence may have caused the difference in transcription activity. Another difference in AGF25 and AG is two nucleotides immediately upstream of the *chimeric-uidA* initiation codon. In case of AG, the two nucleotides were substituted from the wild-type TT to CC, to generate the restriction enzyme site for the construction the *chimeric-uidA*. On the other hand, the two nucleotides were unchanged in the *chimeric-uidA* for AGF25. Such substitution may have altered the translation efficiency and brought the differential protein accumulations.

CHAPTER IV

Effect of translation on mRNA stability

Introduction

The abundance of chloroplast mRNAs changes in response to developmental and environmental signals (Mullet, 1988). This control is determined by the rate of either synthesis or degradation of mRNA. A number of experiments in higher plants showed that the transcription rate of many plastid genes changes during the development, or the light-dark period. For example, the total transcription rate in the plastid changed during a leaf development in spinach (Deng et al., 1987). In tomato fruit, transcriptions of plastid photosynthetic genes were increased during the night and early morning (Piechulla and Gruissem, 1987). In barley, etiolated seedlings showed the transient increase in the plastid gene transcription when seedlings were shifted into light (Klein et al., 1988). The transcription of the *psbD-psbC* operon in barley was induced by light (Sexton et al., 1990). The effect of light on transcription was also examined in *C. reinhardtii*. For instance, transcription rates of several genes were fluctuated in different time points grown under a 12 hr light/12 hr dark cycle. A light-entrained circadian rhythm accounts for this oscillation (Salvador et al., 1993).

Although transcription rates certainly change in some cases, the change is not always correlated with the fluctuation of the mRNA accumulation. During the light-dependent chloroplast development and the leaf maturation in spinach, the mRNAs from the 10 spinach genes were accumulated at different levels, whereas their relative rates of transcription had no major changes (Deng and Gruissem, 1987; Deng et al., 1987). In *C. reinhardtii*, the changes in transcription rates and the mRNA levels of various transcripts were not parallel during the light and dark periods (Salvador et al., 1993). Moreover, the experiment in the spinach leaf development showed that the stability of the *psbA* mRNA was increased more than 2-fold, while the stability of the *rbcL* mRNA did not changed (Klaff and Gruissem, 1991). The result was consistent to the differential mRNA accumulations in the *psbA* and *rbcL* during the development in spinach cotyledons (Deng and Gruissem, 1987). These results suggest that the differential mRNA accumulation is regulated at the level of post-transcription in some extents. The control of the gene expression by the level of post-transcription may have an advantage in which cells can respond to the change of external signals more quickly toward translation than the control at the level of transcription.

The differential mRNA stability in the chloroplast have been supposed to be regulated by both *cis*-element on the mRNA and *trans*-acting factors (for reviews, Gruissem and Schuster, 1993; Mayfield et al., 1995; Rochaix, 1996; Sugita and Sugiura, 1996). Most transcripts from chloroplast genes contain inverted repeat (IR) sequences at their 3'-UTRs. IRs have the

potential to form the stem-loop secondary structure (Gruissem and Tonkyn, 1993). In spite of the structural similarity with transcription terminators in prokaryotes, the 3'-UTRs of the chloroplast mRNAs do not serve as efficient transcription terminators (Blowers et al., 1993; Rott et al., 1996; Stern and Gruissem, 1987). The 3'-UTR rather plays a role in mRNA end processing. In *C. reinhardtii*, deletions in the *atpB* 3'-UTR affected the mRNA stability (Stern et al., 1991). Introduction of the *atpB* 3'-UTR into the chloroplast genome in the sense or antisense orientation showed that both the nucleotide sequence and stem-loop structure of the 3'-UTR are important for 3'-end processing and for the accumulation of the mature mRNAs (Rott et al., 1998). In spinach, progressive deletions within the IR of the *petD* diminished its mRNA stability *in vitro* (Stern et al., 1989). IR-containing mRNA precursor was cleaved at downstream from the mature terminus by an endonucleolytic activity, followed by an exonucleolytic processing in a 3' to 5' direction, generating 3'-ends close to the IR (Hayes et al., 1996). Mutations within the IR structure of the *psbA* mRNA enhanced its mRNA stability *in vitro* and *in organello* (Adams and Stern, 1990). These results suggest that the 3'-UTR is critical for mRNA stability. In addition, RNA-binding proteins, involving in the 3'-UTR-protein complex, were analyzed in tobacco (Ye et al., 1991) and spinach (Hayes et al., 1996; Schuster and Gruissem, 1991). In the absence of these proteins, the 3'-end processing was prevented and the mRNA was degraded. Five RNA-binding proteins were isolated in tobacco, which share two conserved RNA-binding domains and preferentially bind to U- or G-rich stretches in RNA molecules (Li and Sugiura, 1990; Ye and Sugiura, 1992). A 28 kDa protein appeared to be involved in the 3'-end processing in spinach (Schuster and Gruissem, 1991). The proteins, ranging from 28 kDa to 33 kDa in tobacco and the 28 kDa protein in spinach, were all determined as nuclear-encoded proteins, implying that the stability of the chloroplast mRNA is regulated by nucleus. Together with the RNA-binding protein as a *trans*-acting factor, the 3'-UTR may acts as a *cis*-element and protect mRNAs from nucleases, and even acts as a recognition site for degradation. While the 3'-UTR has been focused in the most studies, the 5'-UTR and 5'-UTR binding protein also play important roles in the mRNA stability (Chapter II, Introduction).

Along with UTRs and RNA-binding proteins, effect of polyadenylation on the mRNA stability has been focused recently. Polyadenylation had been thought to stabilize the mRNA and be distinctive in eukaryotes (Manley, 1995). Nevertheless, polyadenylate polymerases were isolated both from *E. coli* and the chloroplast (Burkard, 1974). On the other hand, the elimination of polyadenylate polymerases stabilized the some mRNA species in *E. coli* (O'Hara et al., 1995). In deed, in *E. coli*, many mRNAs have poly (A) tails, ranging from 10 to 50 nt long (Cohen, 1995). Similarly, the polyadenylated *petD* mRNA was found in the chloroplast of spinach. Polyadenylation appeared to promote the efficient degradation of processed *petD* mRNA by a 3' to 5' exoribonuclease (Kudla et al., 1996). In addition, several polyadenylation sites were found in the coding sequence and mature end of the *psbA* mRNA (Lisitsky et al.,

1996). Moreover, the polyadenylation inhibitor stabilized the *psbA* and *rbcL* transcripts in the chloroplast extract from spinach (Lisitsky et al., 1997). These results suggest that post-transcriptional polyadenylation destabilizes mRNAs in bacteria and in the chloroplast.

The regulation of mRNA stability in the chloroplast has been elucidated as described above. However, how or when mRNAs are degraded remains ambiguous. Recently, translation has been focused in the degradation mechanism. The growing evidence suggest that translation is a critical determinant for mRNA stability in the chloroplast. For example, *C. reinhardtii* mutants defective in translation showed the different amount of the mRNA to the wild-type (Drapier et al., 1992; Kuchka et al., 1989; Xu et al., 1993). In spinach leaves, the polysome assembly affected the kinetics of the *psbA* and *rbcL* mRNA degradation (Klaff and Gruissem, 1991).

In this chapter, the relationship between translation and mRNA stability was examined by the *uidA* reporter analysis. In Chapter III, the accumulation of the *chimeric-uidA* in AGF25 was approximately 4-fold lower than in AG. On the contrary, the GUS protein accumulation in AGF25 was approximately 4-fold higher than in AG. I proposed that the mutation of the two nucleotides immediately upstream of the *chimeric-uidA* start codon as a candidate for the incoodination between AGF25 and AG. Here, I confirmed this hypothesis by the site-directed mutagenesis and showed that the translation efficiency affects the mRNA degradation. In addition, half-lives and the accumulation of the *chimeric-uidA* transcript were increased in the presence of a translation inhibitor. The accumulations of mRNAs from several endogenous chloroplast genes were also increased by the translation inhibition. These result suggest that translation destabilizes the chloroplast mRNAs.

Materials and Methods

Strain and culture conditions

C. reinhardtii wild-type 137c and transformants were grown in TAP or M medium under the light or in the dark at 28°C. Where necessary, the medium were solidified with 2 % agar and supplemented with spectinomycin. Cells were harvested at about 2×10^6 cells/ml for all RNA isolations and protein isolations except *in vivo* labeling studies.

DNA constructs

Recombinant DNA plasmids were prepared by conventional procedures. Bacterial host was *E. coli* DH5α.

A 2.4 kb *Cla*I-*Kpn*I fragment from pGatpA was cloned into pBluescript II SK$^+$ (Stratagene) to create plasmid pGAck. A 1.9 kb *Xho*I-*Hin*dIII fragment from pGatpA was cloned into pBluescript II SK$^+$ to create plasmid pGAxh. Using Transformer Site-Directed Mutagenesis Kit (Clonetech), two nucleotides substitution was promoted to the pGAxh with an oligonucleotide

AGS2 as the mutagenic primer (Table 4-1), generating plasmid pGASxh. A 0.5 kb *Xho*I-*Sna*BI fragment from pGASxh was cloned into *Xho*I-*Sna*BI digested pGAck to create pGASck. A 2.4 kb *Cla*I-*Sph*I fragment from pGASck was cloned into *Cla*I-*Sph*I digested pGatpA. Resulting plasmid was designated pGatpAS.

For the mRNA analysis, the coding sequences of the chloroplast genes were amplified by PCR with total DNA from wild-type cells as template. PCR primer pairs were ATPB5 and ATPB3 for a 1.5 kb coding sequence of the *atpB*, PETD5 and PETD3 for a 0.5 kb of the *petD*, TUFA5 and TUFA3 for a 1.3 kb of the *tufA* (Table 4-1). Each PCR product was subcloned into the plasmid vector pUC19. Resulting plasmids were designated pUCatpB, pUCpetD, and pUCtufA, respectively. The plasmids pUC16S, pUCrbcL, pUCpsbA, pUCatpA, and pUCGUS were described in Chapter II.

Analysis of the *chimeric-uidA* gene expression

Individual steps of the procedure were followed essentially as described in Chapter II.

Determination of a half-life of the mRNA

For the determination of the mRNA half-life, transformant cells were labeled with ^{32}P-orthophosphate for 20 min. The labeled cells were washed, resuspended in a high-phosphate (15 mM) TAP medium at a density of about 2×10^7 cells/ml. The suspensions were divided and incubated on a shaker under the light with or without chloramphenicol. Samples (2 ml) were collected at different time points from flasks, centrifuged, and frozen in liquid nitrogen prior to the RNA extraction. After the hybridization with mRNA specific probes, the filters were exposed to an imaging plate (Fuji Photo Film), and the signals were quantified by a bio-imaging analyzer BAS 2000 (Fuji Photo Film). The data were plotted semilogarithmically and a half-life of the mRNA was calculated with a linear regression algorithm for $y = ax + b$.

Inhibition of protein synthesis

Chloramphenicol at a final concentration of 500 μg/ml was added to the cell culture to inhibit protein synthesis in the chloroplast. Where necessary, cycloheximide at a final concentration of 10 μg/ml was added at the same time.

Analysis of *in vivo* protein labeling

Cells grown in TAP medium to about 2×10^6 cells/ml were washed and resuspended in no sulfate TAP medium at a density of about 1×10^7 cells/ml. Cultures were shaken for 2 hr and the protein synthesis inhibitor was added for 5 min. Cells were labeled with carrier-free $Na_2[^{35}S]SO_4$ (ICN) for 15 min as described previously (Takahashi et al., 1994). Aliquots of cultures were centrifuged and cell pellets were frozen at -80°C. Subsequently, the total cell

proteins were separated by SDS-PAGE with a 15% acrylamide gel. The gel was exposed to a X-ray film with an intensifying screen at -80°C.

Table 4-1. Oligonucleotides used in this work.

oligo:	Sequence (5' to 3')
ATPB5	ATGCCTTGGGGCATATTAATTCC
ATPB3	TTATTTTAATGAAGCAGCTTTACTAATAG
PETD5	ATGTCAGTTACTAAAAAACCTG
PETD3	TTAGAATAAACCTAAAGTTAAAG
TUFA5	ATGTCACGTGCTAAGTTTGAACG
TUFA3	TTATTGAACAATATTAGTTACAACACC
ATPS5	ATTTTTTCTTTTTTATGGTACGTCCTGTAG

Results

The mutations in the upstream of the *chimeric-uidA* initiation codon.

In Chapter III, the mRNA accumulation of the *chimeric-uidA* in AGF25 was approximately 4-fold lower than in AG. On the contrary, GUS protein accumulation in AGF25 was approximately 4-fold higher than in AG. The difference in transcription activities between AGF25 and AG was not significant. The mutation of the two nucleotides immediately upstream of the *chimeric-uidA* initiation codon may cause the differences in the gene expression between AGF25 and AG (Chapter III, Discussion).

To confirm this hypothesis, site-directed mutagenesis was promoted to the nucleotides immediately upstream of the *chimeric-uidA* initiation codon in AG, changing from CC to TT which are the same as in AGF25 (Fig. 4-1). The derived transformant was designated AGS. The only difference between AGS and AG was this two nucleotides substitution. As a result, the *chimeric-uidA* transcripts and proteins produced in these transformants should be identical.

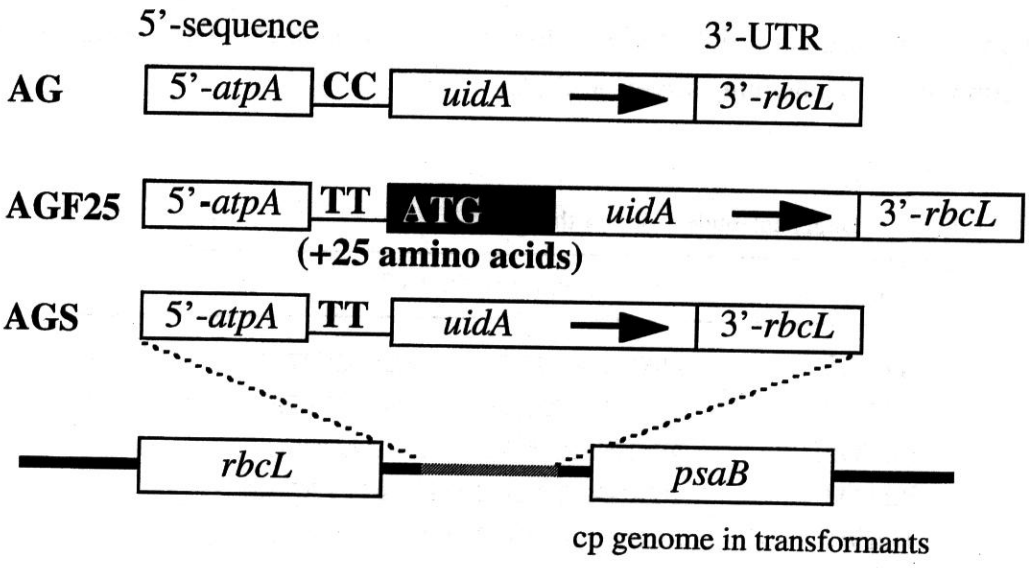

Figure 4-1. Transformants harboring different *chimeric-uidA*s. Each *chimeric-uidA* contains a putative promoter and a 5'-UTR from the *atpA*, *uidA* coding sequence, and the 3'-UTR from the *rbcL*. AGF25 carries the portion of the *atpA* coding sequence (see Chapter III). Two nucleotides immediately upstream of the translation initiation site are CC in AG, or TT in AGF and AGS.

No significant difference was observed in transcription activities of the *chimeric-uidA*s between AGS and AG (Fig. 4-2, with the *uidA* probe).

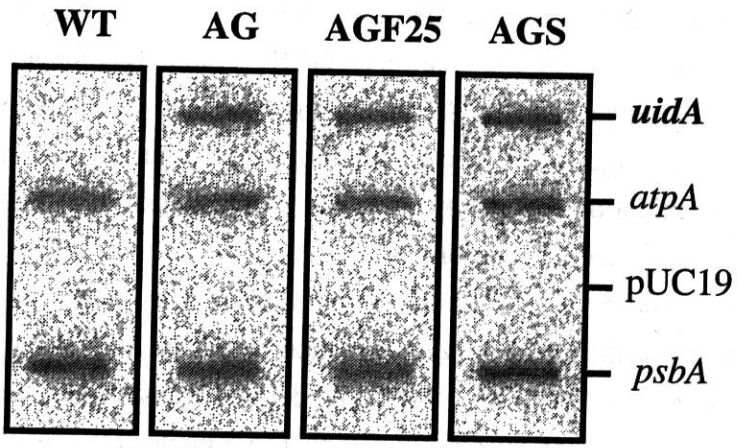

Figure 4-2. Transcription activity of the *chimeric-uidA* in each transformant. Total RNA, labeled *in vivo* with 20 min pulse of ^{32}P-orthophosphate, was hybridized to nylon filters with immobilized plasmids pUCGUS, pUCatpA, pUCpsbA. A plasmid pUC19 was a probe for a negative control.

On the other hand, the mRNA accumulation in AGS was lower than in AG (Fig. 4-3), while GUS specific activity in AGS was 5.9-fold higher than in AG (Table 4-2).

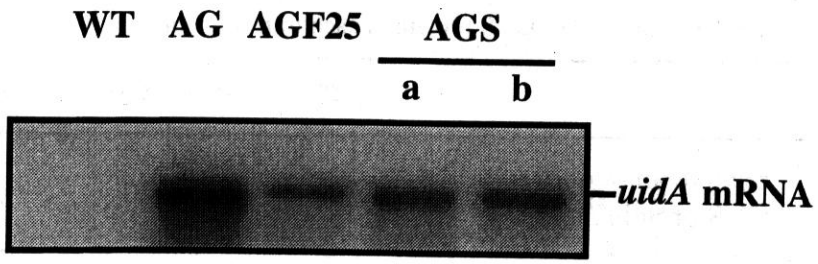

Figure 4-3. The accumulation of the *chimeric-uidA* mRNA in each transformant. Total RNA (5 μg) extracted from TAP grown cell culture was hybridized with a 1.8 kb *NcoI-SphI* fragment of the pT7GUS. *C. reinhardtii* wild-type 137c (WT) and transformants AG, AGF25, and two independent clones of AGS (a, b) were analyzed.

Transcription activity (Fig. 4-2) and the accumulation of the *chimeric-uidA* transcript (Fig. 4-3) were comparable in AGF25 and AGS. Although the enzyme activity in AGF25 was 2.2-fold lower than in AGS (Table 4-2), the accumulation of the protein examined by western analysis appeared to be unchanged (Fig. 4-4 and Table 4-3). A portion of α subunit of ATP synthase fused to the GUS protein may negatively influence to the enzyme activity, as observed in the *rbcL-uidA* transformants in Chapter III. Therefore, I conclude that the difference observed between AGF25 and AG was also caused by the two nucleotides substitution as in the case of AGS and AG. In the later experiments, I compared AGF25 with AG, instead of AGS with AG,

Table 4-2. GUS specific activity in each transformant.

	AG	AGF25	AGS
GUS specific activity [a)] (nmole/min/mg protein)	410 ± 26	1100 ± 100	2400 ± 250

a) Cells were grown in the TAP medium. Mean (± SD) of three independent experiments.

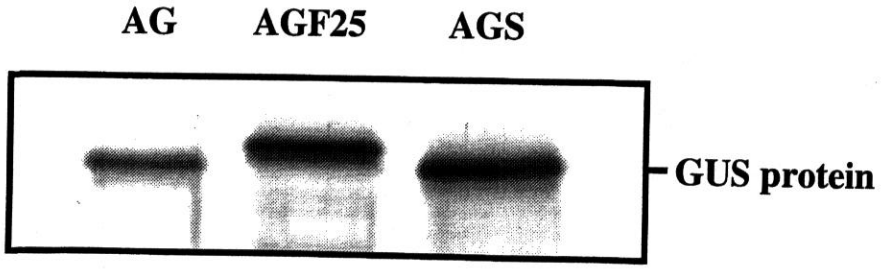

Figure 4-4. Western analysis of crude extracts. Protein extract (1 μg) from the cells grown in TAP medium were separated by 7.5% acrylamide SDS-PAGE, and GUS proteins were detected by western blotting.

Table 4-3. Relative amount of GUS protein in each transformant.

	AG	AGF25	AGS
relative amount of GUS protein[a] (%)	1.00	2.20	2.66

a) Relative amount of the GUS protein in the transformant to the amount in AG.

Stability of the *chimeric-uidA* transcripts *in vivo*.

In AGF25 and AG, transcription activities of *chimeric-uidA*s appeared to be unchanged, whereas the difference in the accumulation of the mRNAs was apparent. The result suggested that mRNA stabilities in both transformants were different. To determine the half-lives of the *chimeric-uidA* transcripts in AGF25 and AG, the decay of the mRNAs was measured in *in vivo* pulse and chase experiment. The result showed that the decay of the mRNA in AGF25 was relatively faster than in AG (Fig. 4-5A). Under my experimental conditions, the *chimeric-uidA* transcript was decayed with approximate half-lives of 37 min and 63 min in AGF25 and AG, respectively (Fig. 4-7, Table 4-4). The decay rate of 16S rRNA, as an internal control, appeared to be unchanged in both transformants. As expected, stabilities of *chimeric-uidA* mRNA were different between AGF25 and AG. Comparing with AG, the signal of labeled transcript in AGF25 was lower at 0 min, the start point of the chase experiment. Since the decay rate in AGF25 was almost 2-fold faster than in AG, the period between the termination of the label and beginning of the chase may be long enough to cause this reduction.

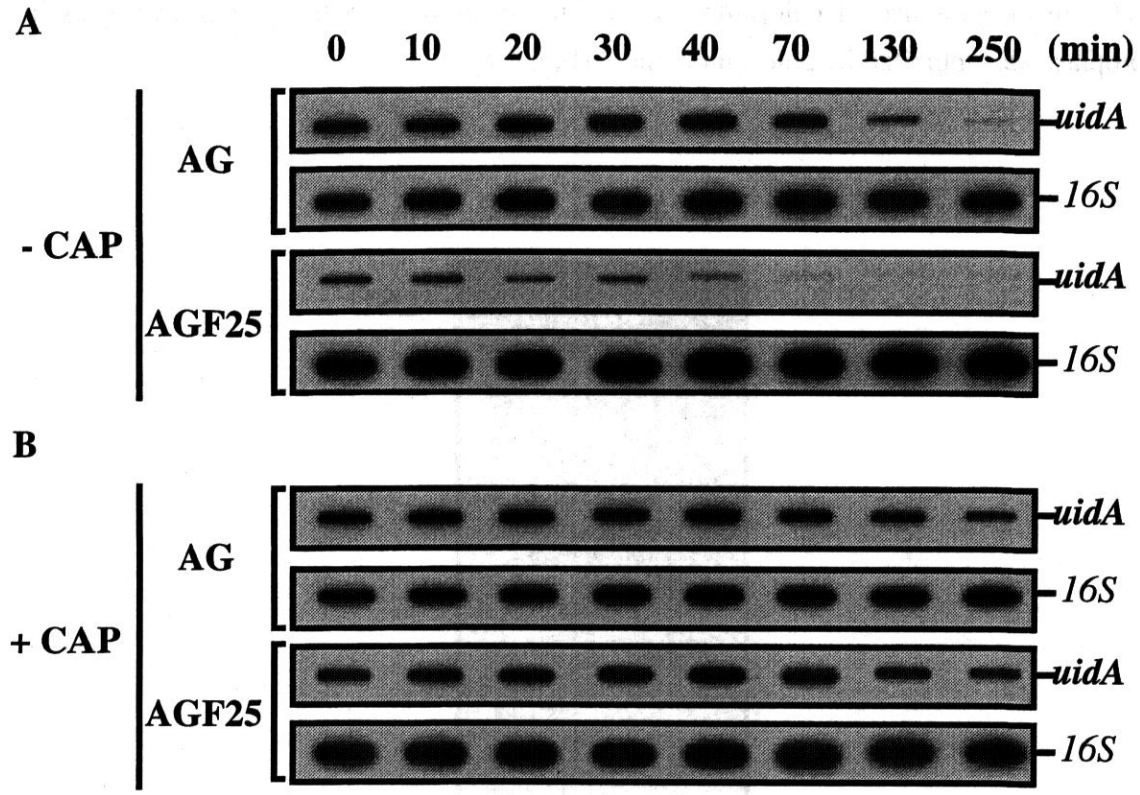

Figure 4-5. *In vivo* pulse and chase analysis for the *chimeric-uidA* decay in transformants. Total RNA was labeled with 20 min pulse of ^{32}P-orthophosphate, and chased for different time points in the absence (A: - CAP) or presence (B: + CAP) of chloramphenicol. Total RNA was hybridized to nylon filters with immobilized plasmids pUCGUS and pUC16S.

Effect of the translation inhibition on mRNA stability.

The mutation analysis suggests that the mutation changed translation efficiency, and consequently, the *chimeric-uidA* mRNA level was decreased faster in AGF25 compared to AG. If the hypothesis is correct, then the translation inhibition should affect the mRNA decay. Therefore, the effect of an organelle-specific translation inhibitor on mRNA stability was analyzed. Chloramphenicol was chosen as the inhibitor because of its remarkable permeability (Vazquez, 1979). Chloramphenicol interacts with 50S ribosomal subunit and blocks peptide bond formation. This inhibitor especially effects on the peptidyl transferase center of RNA-bound ribosomes and stalls elongating ribosomes on the mRNA (Vazquez, 1979). First, to confirm the inhibition of protein synthesis, newly synthesized proteins were labeled with [^{35}S]SO^{2-} by *in vivo* labeling, in the absence or presence of chloramphenicol. To focus on the chloroplast translation, the cytoplasmic translation was inhibited by cycloheximide. Although the synthesis of an approximately 50 kDa protein was not inhibited by chloramphenicol and cycloheximide, all other signals including the one for the RuBisCO large subunit were not

detected in the presence of chloramphenicol. The result showed the protein synthesis in the chloroplast was inhibited by chloramphenicol (Fig. 4-6).

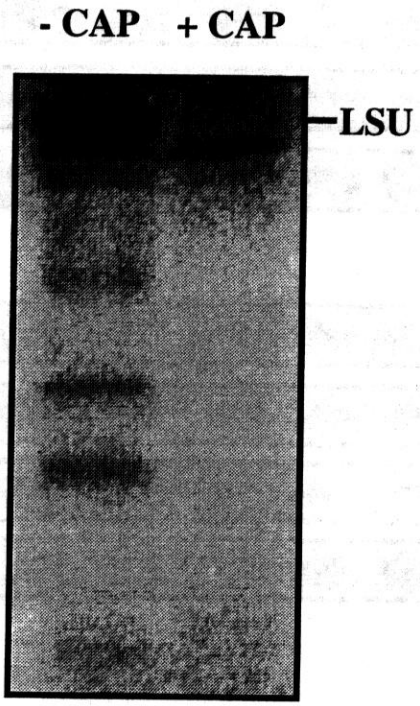

Figure 4-6. Inhibition of protein synthesis in the chloroplast. Cells grown in TAP medium were resuspended in no sulfate TAP medium in the absence (- CAP) and presence (+ CAP) of chloramphenicol and labeled with carrier-free $[^{35}S]SO4^{2-}$ for 15 min. The total cell proteins were separated by SDS-PAGE with a 15% acrylamide gel which was exposed to a X-ray film. LSU represents RuBisCO large subunit.

Then, the mRNA decay was analyzed by the same way as described in Figure 4-5A, except with chloramphenicol in the chase period (Fig. 4-5B). The labeled mRNA levels of the *chimeric-uidA* in AGF25 and AG were increased at each time point. Indeed, the mRNA levels were almost equivalent in both transformants in the presence of the inhibitor. The half-lives of the transcripts were 167 min and 158 min in AGF25 and AG, respectively (Fig. 4-7, Table 4-4). These results suggest that translation affects mRNA stability, and translation was involved in differential mRNA stabilities between AGF25 and AG.

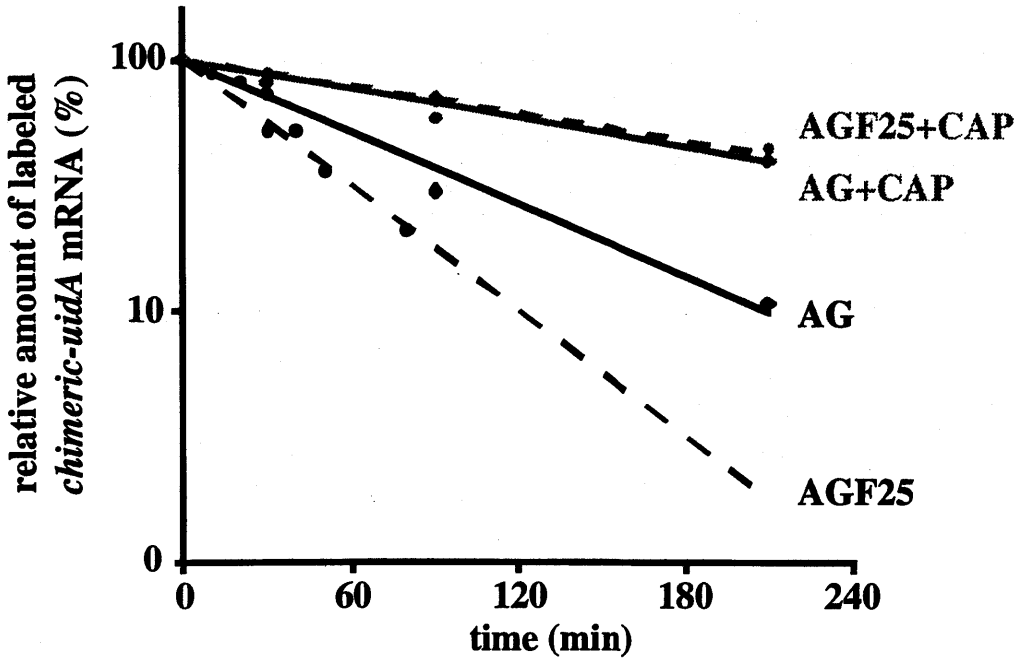

Figure 4-7. Decay of the *chimeric-uidA* in transformants. Labeled RNAs were chased in the absence (AG and AGF25) or presence (AG+CAP and AGF25+CAP) of chloramphenicol (Fig. 4-5). Signal intensities were quantified, and plotted semilogarithmically. The data were subjected to a linear regression algorithm for y = ax + b.

Table 4-4. Half-lives of *chimeric-uidA* mRNAs.

	AG	AGF25	AG+CAP	AGF25+CAP
half-life[a)] (min)	63	37	158	167

a) Half-lives were calculated from a linear regression algorithm for y = ax + b (Fig. 4-7).

The accumulation of *chimeric-uidA* transcripts in the presence of the translation inhibitor.

The effect of translation inhibition on the accumulation of the *chimeric-uidA* mRNA was also examined. Transformant cell cultures were treated with chloramphenicol, and samples were taken at the different time points. Fig. 4-8 showed the result from northern analysis of AGF25 and AG. In AGF25, the accumulation of the *chimeric-uidA* transcript was increased within 5 min after the inhibition treatment. After 120 min, the accumulation in AGF25 reached at the maximum level and the level remained to be unchanged for 240 min (data not shown). The result suggest that translation affects the accumulation of the mRNA. In AG, little change was observed and the mRNA accumulation at 120 min was comparable to the accumulation in AGF25 at the same period.

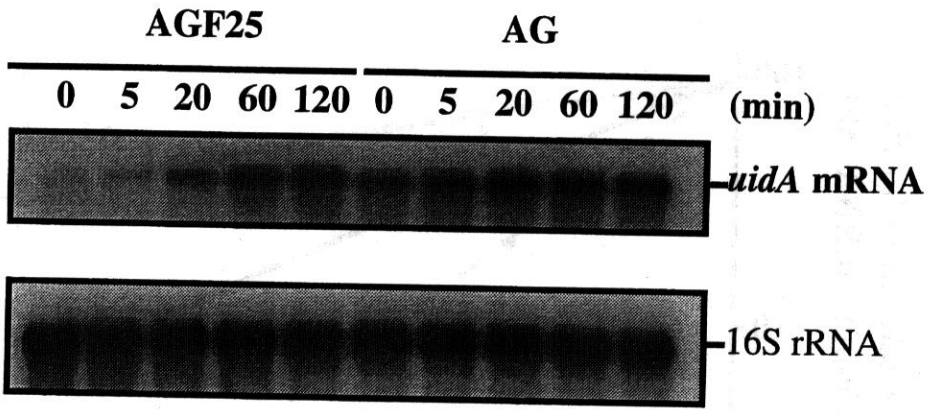

Figure 4-8. Effect of translation inhibition on the *chimeric-uidA* mRNA accumulation. Cell cultures (AGF25 and AG) were treated with chloramphenicol, samples were then taken at 0 min, 5 min, 20 min, 60 min, and 120 min. Total RNA (5 μg), extracted from the cells, was analyzed by northern hybridization.

The accumulation of transcripts from endogenous genes in the presence of the inhibitor.

Then, effects of the translation inhibition on the accumulation of mRNAs from endogenous chloroplast genes was examined. As described in Fig. 4-8, AGF25 cells were treated with chloramphenicol, and samples were taken at the different time points. Northern analysis showed that the translation inhibition increased the accumulation of transcripts from the *atpA*, and from the *tufA* which encodes the elongation factor EF-Tu (Fig. 4-9). On the other hand, the accumulation of transcripts from the *atpB*, *petD*, *psbA*, and the *rbcL* appeared to be unchanged. These results showed that translation inhibition also affects the accumulations of the mRNAs from several endogenous genes.

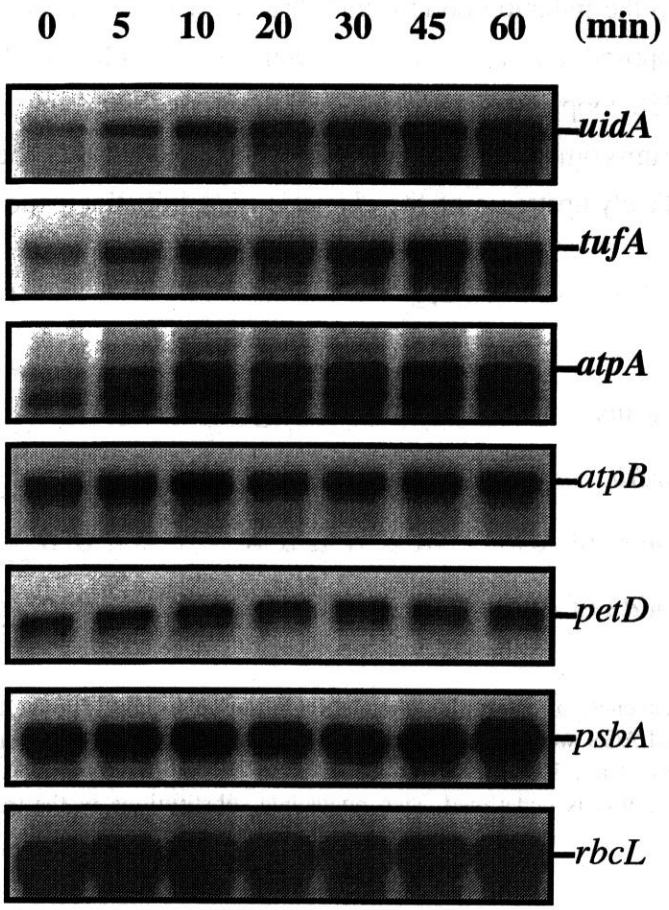

Figure 4-9. Effects of translation inhibition on transcripts from endogenous chloroplast genes. Total RNA (5 μg), extracted from AGF in Fig. 4-8 was hybridized with the coding fragment of the *tufA*, *atpA*, *atpB*, *petD*, *psbA*, and the *rbcL*. The *atpA* probe hybridized to two bands; the larger RNA corresponds to the 2.2 kb *atpA* transcript (Salvador, 1993).

Discussion

Two nucleotides immediately upstream of the initiation codon affect the translation efficiency.

Only the difference between AGS and AG is the nucleotides substitution immediately upstream of the *chimeric-uidA* initiation codon: the nucleotides are TT for AGS and CC for AG (Fig. 4-1). As expected, transcription activities of the *chimeric-uidA*s showed no significant changes between AGS and AG (Fig. 4-2). Therefore, I conclude that the two nucleotides substitution does not affect the transcription. On the other hand, the specific activity of GUS in AGS was 5.9-fold higher than the activity of AG. The site-directed mutagenesis near the initiation codon of the *petA*, encoding cytochrome *f*, resulted in the change of the translation efficiency (Higgs et al., 1997). A sequence comparison of the 37 chloroplast genes in *C. reinhardtii* suggests that several nucleotides neighboring to the initiation codons are strongly conserved (Fig. 4-10). Moreover, the interaction between the nucleotides at the -1 to -3

position, relative to the initiation codon, and three nucleotides within the 530 loop of the *16S rRNA* has been proposed in *E. coli* (Lagunez-Otero, 1993). mRNA-rRNA cross-linking studies showed that the 530 loop interacts to the region of the transcript near the initiation codon (McCarthy and Brimacombe, 1994). Therefore, I concluded that the substitution of the two nucleotides immediately upstream of the *chimeric-uidA* initiation codon changed the translation efficiency.

	-12	+1
	*	*
vascular plants	G G G G a g a u c A U U A U G G C
C. reinhardtii	a a a u u a u u A u U U A U G G C
chimeric-uidA of AGS	a a a u u a u u A u **U U** A U G G T
chimeric-uidA of AG	a a a u u a u u A u **C C** A U G G T

Figure 4-10. Sequences of chloroplast translation initiation regions. The sequences of the *chimeric-uidA* in AG and AGS were compared to the consensus sequences of the vascular plant and of *C. reinhardtii* (Stern et al., 1997). Nucleotides showing a strong preference are in capital letters, and AUG initiation codon is underlined. Two nucleotide substitutions in the transformants are shown in bold letters.

Translation destabilizes the transcript in the chloroplast.

The mRNA accumulation of the *chimeric-uidA* in AGF25 was 4-fold lower than in AG (Fig. 4-3), and the western analysis implied that translation efficiencies in AGF25 and AG appeared to be different (Fig. 4-4), as is the case in AGS and AG. The result suggests that translation plays a role in mRNA stability. To confirm this hypothesis, I determined half-lives of *chimeric-uidA* transcripts and examined the effect of translation on mRNA stability. The mRNA in AGF25 was decayed approximately 2-fold faster than the transcript in AG, with half-lives of 37 min and 63 min in AGF25 and AG, respectively (Fig. 4-5A and Table 4-4). mRNA stabilities were consistent with the accumulations of the transcripts. On the other hand, in the presence of chloramphenicol, half-lives of the transcripts were calculated as 167 min and 158 min in AGF25 and AG, respectively (Fig. 4-5B and Table 4-4). Lincomycin, another translation elongation inhibitor, also demonstrated a similar effect (data not shown). In Chapter III, another possibility that may cause the incoodination in the accumulations of mRNAs and proteins between AGF25 and AG was discussed. A part of the *atpA* coding sequence per se, or the change in the secondary structure of the *chimeric-uidA* would have the effect on the mRNA stability. Nevertheless, mRNA degradation kinetics of both AGF25 and AG were comparable in the translation inhibition experiment, suggesting that the differential mRNA stabilities are depending on translation. Therefore, I assume that the differential mRNA accumulations in AGF25 and AG were not caused by the fusion of the 5'-coding sequence, but caused by the change of the

translation efficiency. From these results, I concluded that translation destabilized the *chimeric-uidA* transcript in the chloroplast.

The translation inhibition itself is the reason for the increase of the mRNA stability.

It is possible that the effect of the translation inhibition on mRNA stability and accumulation was due to the depletion of proteins, which destabilize the chloroplast mRNAs, presumably RNases. Chloramphenicol does not inhibit the protein synthesis in nucleus (Vazquez, 1979), so that the depletion of the nuclear-factors should not account for the change of the mRNA stability and accumulation. Still, during the chloramphenicol treatment, the synthesis of unknown chloroplast-encoded destabilizing proteins might have been inhibited. Turnover of such proteins might affect mRNA stability. However, the response of mRNA accumulation to the translation inhibitor was observed within 5 min (Fig. 4-8). Partaking of such extremely short-lived protein in this mRNA degradation is inconceivable. In addition, chloroplast genome does not contain the sequence homologous to the genes for RNases. Therefore, the increases of the mRNA stability and its accumulation were not due to the proteins depletion, but due to such translation inhibition itself.

Co-translational mRNA degradation in the chloroplast of *C. reinhardtii*.

The role of translation on mRNA stability has been described for decades in prokaryotes and eukaryotes. In *E. coli*, antibiotics such as chloramphenicol, tetracycline, and puromycin, all interfere the polypeptide elongation, changed the stability of total mRNAs in the cell and mRNAs from individual genes (Petersen, 1993). The mRNA degradation is initiated by RNase E cleavage (Cohen and McDowall, 1997) and translation inhibits this degradation by protecting the transcript from the RNase E (Braun, 1998). Also, the polysome association regulates the stability of mRNAs from genes for β-lactamase and outer membrane protein A in *E. coli* (Nilsson et al., 1987), and mRNAs for mammalian β-globulin and for the yeast MATα1 (Parker and Jacobson, 1990). In eukaryotes and prokaryotes, translation is generally thought to stabilize the transcripts. However, translation seems to rather destabilize the mRNA in some cases. For example, cycloheximide, a cytoplasmic translation elongation inhibitor, blocked the degradation of tublin mRNA (Gay et al., 1989; Gong and Brandhorst, 1988). Translation inhibition also stabilized histone mRNA, and the transcripts from the *c-myc* and *c-fos* (Laird-Offringa et al., 1990; Linial et al., 1985; Stimac et al., 1984; Wilson and Treisman, 1988). All these mRNAs carry instability *cis*-elements. A number of the premature translation termination have also been reported to increase the mRNA stabilities (Theodorakis and Cleveland, 1996).

Effect of translation on mRNA stability have also been predicted in the chloroplast. The experiment with chloramphenicol or lincomycin showed the polysome assembly affected the kinetic of the *psbA* or *rbcL* mRNA degradation in spinach leaves. The inhibition also

demonstrated the translation was critical for the differential turnover of both mRNAs (Klaff and Gruissem, 1991). In *C. reinhardtii*, the nuclear mutant defective in translation of the *psbD* or *atpA* showed the transcript level of each gene was 2 to 4-fold over the wild-type amount (Drapier et al., 1992; Kuchka et al., 1989). The chloroplast mutant cc2341, a single base pair deletion caused a premature termination of the *psaB*, encoding II subunit of PS I protein, showed that the level of the *psaB* mRNA was increased approximately 2.5-fold relative to the wild-type cell (Bingham et al., 1991; Xu et al., 1993). These previous works are consistent with my results that showed translation destabilized the transcripts.

As described above, mutants of *C. reinhardtii* have provided us the fruitful results. However, the results are sometimes confusing. For example, in the nuclear or chloroplast mutation that prevents the translation of the *psbC*, the mRNA stability was not changed (Rochaix et al., 1989). The *psbD* mRNA transcript was destabilized in the chloroplast mutant FuD47, which contains a frame-shift mutation in the *psbD* gene (Erickson et al., 1986), indicating that translation stabilizes the mRNA. These results suggest that the effects of translation on mRNA stability are depending on the genes. This implication is consistent with the result of my northern analysis in which the translation inhibition affected differently the accumulations of the mRNA from endogenous chloroplast genes. Or, differential results might have been a side effect of the mutation. Two mutants, each contains the mutation even in the same chloroplast gene, demonstrated the different result. The mutant 18-7G, containing a nonsense mutation close to the 5'-end of the *rbcL* gene, accumulated a normal level of the transcript. On the other hand, the mutant 18-5B, containing the nonsense mutation in the 3'-end, the transcript was decreased (Spreitzer et al., 1985). The mutation usually affects photosynthesis and may change the over-all gene expression in the chloroplast.

The model for the mechanism of co-translational mRNA degradation.

Along with the previous results, I present the following models for co-translational mRNA degradation: (1) Ribosomes or other parts of the translational apparatus are associated with nucleases, or with factors that recognizes the instability determinants. Degradation is initiated when the translation elongation begins. Biochemical approaches are necessary to confirm the presence of the nucleorytic activity in the polysome-bound mRNA fraction. The disruption of translation initiation codon, or the application of antibiotics which inhibit the translation initiation, would be helpful to elaborate the role of ribosomes. This hypothesis may not explain the gene specificity for the degradation (Fig. 4-11A). (2) The ribosome carries a nuclease or nuclease-activating protein which recognizes the mRNA 3'-UTR and initiates the degradation. If the ribosome falls off the mRNA before reaching the 3'-UTR, then the mRNA is not degraded. This hypothesis can be applicable to the cases of several mutants which contain the premature translation termination (Fig. 4-11B). (3) A portion of peptides, translated from the mRNA, binds to the factor that initiates mRNA degradation (Fig. 4-11C). (4) Translation alters the

secondary structure of the mRNA, which makes the nuclease accessible to the mRNA. (5) Translation localizes the RNA to a organellar region where the degradation machinery exists. (6) Ribosome or the translational apparatus removes the protein, which binds to the 3'-UTR and prevent the mRNA from degradation. In *E. coli*, the ribosome masks 10 to 20 nt from the stop codon (Braun, 1998). Therefore, providing that the protein binds to the mRNA in no less than 20 nt distance from the UAA stop codon, the ribosome can reach the protein before being released from the mRNA at the stop codon. The elimination of these proteins destabilizes the mRNA (Fig. 4-11D). A number of mutagenesis which introduce the stop codons into the several locations on the coding sequence may let us confirm this hypothesis.

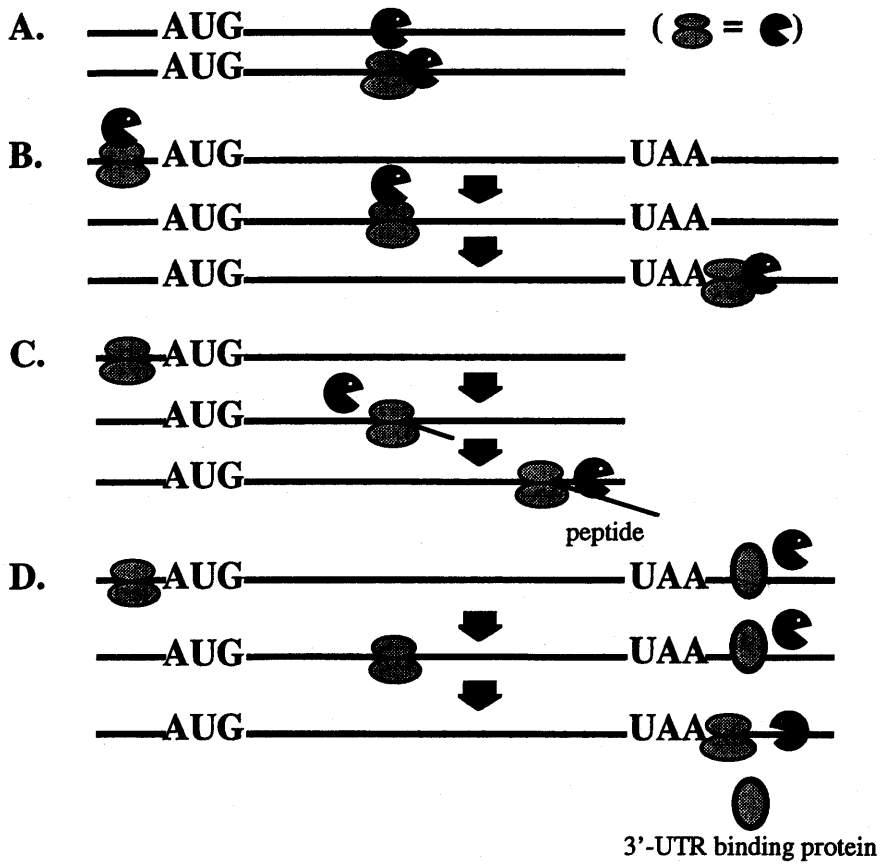

Figure 4-11. Hypothetical mechanisms for the co-translational degradation of the chloroplast mRNA. Packman represents the RNase activity. A. Ribosomes or a translation apparatus is associated with nucleases. B. Ribosomes carry a nuclease or a nuclease-activating protein that recognizes the mRNA 3'-UTR. C. A portion of peptides, translated from the mRNA, binds to the factor that initiates mRNA degradation. D. Translation removes proteins, binding to the 3'-UTR and protecting the mRNA from degradation. The change of the mRNA secondary structure by translation, or differential mRNA localization by translation may also be the reason for the co-translational degradation.

Effects of the translation inhibition differ among chloroplast genes.

Effect of translation inhibition on mRNA accumulations was observed not only in the reporter gene but also in the *atpA* and *tufA* (Fig. 4-9). On the other hand, in other chloroplast

endogenous genes, such as the *rbcL*, *psbA*, *petD*, and the *atpB*, the mRNA accumulations appeared to be unaffected by the translation inhibition. The result may simply suggest that the co-translational mRNA degradation mechanism exists only for some of chloroplast genes. The *cis*-element may play a role in this gene specific mRNA degradation.

Even though this explanation appears to be appropriate, another interpretation is possible. In the chloroplast, transcripts might be naked or protected by RNA-binding proteins, with a monosome or polysomes, and processed or unprocessed, resulting in the total mRNA mixture. Each transcript should act differently in the gene expression, especially at the level of translation. Providing that most of the mRNA molecules do not associate with polysomes, the population of translatable transcripts is quite small relative to the total amount of the mRNA mixture. In such case, even if the co-translational degradation exists for the mRNA, destabilized mRNA molecules would be small enough and the change of the mRNA accumulation is virtually indistinguishable. In fact, more than 80% of the *psbA* mRNA were found in non-polysomal RNA fraction in the chloroplast of *C. reinhardtii* grown in continuous light (Cohen et al., 1998). Moreover, translation efficiencies of the mRNAs from the *atpB*, *rbcL*, and *psbA* are much lower than the efficiency for the *atpA* (Drapier et al., 1992). For such reasons, decreases of these mRNAs by translation would be quite small so that the changes in the accumulations would be virtually unchanged. Therefore, it is possible that the effect of the inhibition on mRNA stabilities of the *rbcL*, *psbA*, *petD*, and the *atpB* would be observed when *in vivo* pulse and chase experiment is applied. In this scheme, co-translational mRNA degradation might be a general mechanism for the chloroplast. This explanation is supported by the results in Figs. 4-7 and 4-8. *In vivo* pulse and chase experiment showed that the translation inhibition well affected the *chimeric-uidA* mRNA stability and increased its half-life by 2.5-fold in AG (Fig. 4-7). On the contrary, the mRNA accumulation was observed to be unaffected by the inhibition in the northern analysis (Fig. 4-8). Since the *chimeric-uidA* mRNA was less translated and more stable in AG than in AGF25, the decrease of the mRNA in AG should be relatively small. This state of the mRNA might have brought the change in the accumulation indistinguishable.

The hypothesis suggests that we have to take into account of the states of the transcript for the effect of translation on mRNA stability. This perspective should be indispensable to understand the chloroplast gene expression, although few works have respected this viewpoint, and none of the examination have shown the relationship between the protein amount and the mRNA accumulation. Also, many works relied mainly on northern analysis. However, the quality of the transcript can not be distinguished by northern analysis, which only shows the quantity of the transcript. Hence, combination analysis of the polysome assembly, mRNA stability, the accumulation of the transcript, and the protein amount in several environmental conditions would provide us the physiological meaning of co-translational mRNA degradation.

CHAPTER V

Conclusion

The analysis of the chloroplast gene expression.

I examined the chloroplast gene expression, at the level of transcription, post-transcription, and translation. The *uidA* reporter analysis was proved to be useful to understand the gene expression as an ensemble of each level. Outcomes were follows: (1) The sequence only contains the putative promoter and 5'-UTR did not always reflect the endogenous gene expression pattern (Chapter II). (2) A portion of coding sequence of the *rbcL* affected transcription, and a portion of coding sequence of the *psbA* affected transcription and mRNA stability (Chapter III). (3) Translation accelerated the mRNA degradation (Chapter IV). In spite of the achievements in this thesis, one should use the reporter analysis carefully, since the reporter gene does not always represent the endogenous gene expression. For instance, the coding sequences of the *rbcL* and *psbA* were required for the transcription activity and mRNA stability as shown in Chapter III. In addition, in my experimental condition, the *chimeric-uidA* containing the promoter and 5'-UTR of the *atpA* did not oscillate its mRNA level in the light-dark cycle, while the level from the endogenous *atpA* gene was fluctuated (data not shown). The coding sequence or the 3'-UTR of the *atpA* may control this fluctuation. In fact, 3'-UTR and 3'-end processing affected the mRNA stability and translation of the *atpB* in *C. reinhardtii* (Rott et al., 1998a; Rott et al., 1998b). Therefore, depending on the purpose, the additional sequence is indispensable to reflect the endogenous gene expression. Conversely speaking, regulatory elements can be determined by the loss of function analysis with the reporter gene. Along with the observation of the endogenous gene expression, the introduction of the *uidA* reporter gene would help us to understand the gene expression in the chloroplast.

The chloroplast genes are expressed through the level of transcription, post-transcription, translation, and post-translation, as is the case in prokaryotes or eukaryotes. Each level is processed in a progressive reaction and each level should act in concert with others for the gene expression. Earlier levels affect the later levels for the expression. Even the later level influences the earlier step. In Chapter IV, the mRNA accumulation did not correlate with the protein accumulation, suggesting that the mRNA is degraded by translation in the chloroplast, and translation affected the earlier step, post-transcription. Not only in the chloroplast, several data show that one of the levels does not always reflect the expression of nuclear genes. Nevertheless, most studies observe only a few of these steps, usually the mRNA accumulation. For understanding of the gene expression, we should examine each step carefully, and consider the effects of each step on the expression more comprehensively.

Effects of the coding sequence on transcription and mRNA stability.

In Chapter II, the 5'-sequences from the *rbcL* and *psbA* fused with the *uidA* did not reflect transcription activities of the endogenous genes. In Chapter III, portion of coding sequences of the *rbcL* and *psbA* enhanced transcription activities of the *chimeric-uidA*s. The portion of the *psbA* coding sequence was also determined as a stabilizing element for the *chimeric-uidA* transcript. Hence, the coding sequence affects the chloroplast gene expression, at least in transcription and post-transcription. On the other hand, a portion of the *atpA* coding sequence did not affect the transcription activity. The different regulations may exist in the chloroplast gene expression, and the regulations may be depending on the gene.

Co-translational mRNA degradation.

I described in Chapter IV that translation destabilized the mRNA in the chloroplast. On the contrary, translation rather stabilized the mRNA in *E. coli* (e.g., the house keeping gene *rpsO* or *lacZ*, encoding 15S ribosomal protein or β-galactosidase, respectively) (Braun et al., 1998; Iost and Dreyfus, 1995). These results suggest that the machinery itself is different between the chloroplast and *E. coli*. Co-translational mRNA degradation may be distinctive for the chloroplast. However, the change of the mRNA accumulation by the translation inhibition was observed in the *atpA* and *tufA*, but not in the *rbcL*, *psbA*, *atpB*, and the *petD*. In addition, several mutants demonstrated that the mRNAs from some chloroplast genes were even increased by translation. These results suggest that co-translational mRNA degradation mechanism exists only for some of the genes, and the different regulations control individual genes. Therefore, that mRNAs from several genes are degraded by translation in *E. coli* can not be denied.

I also referred that the translation inhibition increased the *chimeric-uidA* mRNA stability, but the inhibition appeared not to affect the mRNA accumulation in the AG transformant. The result implies the experimental problem of northern analysis in which only the quantity of the transcript is observed, but the quality of the transcript can not be distinguished. In the chloroplast, transcripts exist in the several states, and each transcript should act differently in the gene expression. Providing that most of the mRNA molecules are not in the polysome-bound fraction, translatable transcripts should be quite small relative to the total amount of mRNAs. Consequently, destabilized mRNAs by translation will be small enough so that the change of the mRNA accumulation is virtually indistinguishable. Considering this hypothesis, the changes of mRNA stabilities by translation inhibition for the *rbcL*, *psbA*, *atpB*, and the *petD* might be observed by *in vivo* pulse and chase experiment. In this case, co-translational mRNA degradation can rather be a general mechanism in the chloroplast. Hence, states of the transcript, influenced by the polysome assembly, and processing that make the molecule functional should be considered to understand the effect of translation on mRNA stability.

The chloroplast as a model for the understanding of translation mechanism.

The molecular process of translation has not been described as a conceptual sense. One of the reason is the numerous factors exist in this complicated system. Also, one has to consider the effect of transcription and post-transcription to translation because of the sequence in the gene expression. Moreover, in the case of *E. coli*, transcripts, as templates of translation, are unstable with half-lives of minutes that makes the manipulation of the experiment difficult. In this sense, the chloroplast will be a good model for translation in prokaryotes because its transcripts are relatively stable with the half-lives of hours, favorable for the experimental manipulations.

In bacteria, transcription and translation are synchronized. On the other hand, the chloroplast contains the large population of ribosome-free transcripts. Because of such divergence, the comparison of translation machinery between the chloroplast and bacteria also seems attractive.

A model for the chloroplast gene expression.

From my data and previous results, I presume the chloroplast gene expression as follows. First, transcription of the genes are enhanced by the environmental signals, and in some cases, the coding sequence enhances transcription activity. A portion of the transcripts is translated soon after transcription, and others go to the mRNA population in which the transcripts exist as the polysome-free fraction and incompetent for translation, resulting the mixture of the different states of the transcripts. This mRNA mixture may contribute to the cells in preparation for the quick response to environmental changes. A polysome-free *psbA* mRNA fraction was immunoprecipitated by antibodies against tobacco chloroplast RNA binding proteins (Sugita and Sugiura, 1996), suggesting that the polysome-free mRNA may be stabilized by the proteins with forming a stable RNA-protein complex. In this stage, a complicated signal network may affect the mRNA stability. Once the environment changes (e.g., shifting from dark to light), ribosomes and translation apparatus bind to the 5'-end of the transcript that exists in the mRNA mixture. The 5'-UTR sequence regulates the translation efficiency (Chapter IV), presumably controlling this ribosomal entry - in general, this step is the rate-limiting in translation. The 5'-UTR binding proteins might also escort this ribosome assembly. Even the 3'-UTR is critical for this step (Rott et al., 1998a), suggesting the interaction between both ends of the transcript. The processing of the 5'-UTR may be initiated at the same time (Bruick and Mayfield, 1998). This synchronization may be the reason why changes in the RuBisCO large subunit synthesis in response to light levels coincide with the changes in processing of the *rbcL* 5'-UTR (Shapira et al., 1997). After the ribosome binding and the processing, the transcript may lose a portion of the 5'-UTR and its binding proteins. Still, the transcript may be stable in this stage since it is protected by ribosome itself or the translation apparatus. When the signal initiates translation elongation - this step is also critical for translation in the chloroplast (Edhofer et al., 1998), ribosome and translation apparatus slide on the transcript. Then, ribosome and translation

apparatus may interrupt the association of the UTRs with binding proteins that protect mRNA from degradation. This interruption can take place at the 5'-UTR, coding sequence, or the 3'-UTR. Accordingly, translation may influence mRNA stability. After translation, the product is modified to be functional. A number of biochemical analysis show this step is also important and should be considered for the comprehensive model of the chloroplast gene expression.

REFERENCES

Adams, C. C., and Stern, D. B. (1990). Control of mRNA stability in chloroplasts by 3' inverted repeats: effects of stem and loop mutations on degradation of psbA mRNA *in vitro*. Nucleic Acids Res 18, 6003-6010.

Baker, E. J., Schloss, J. A., and Rosenbaum, J. L. (1984). Rapid changes in tubulin RNA synthesis and stability induced by deflagellation in *Chlamydomonas*. J Cell Biol 99, 2074-2081.

Bingham, S. E., Xu, R. H., and Webber, A. N. (1991). Transformation of chloroplasts with the *psaB* gene encoding a polypeptide of the photosystem I reaction center. FEBS Lett 292, 137-140.

Blowers, A. D., Bogorad, L., Shark, K. B., and Sanford, J. C. (1989). Studies on *Chlamydomonas* chloroplast transformation: foreign DNA can be stably maintained in the chromosome. Plant Cell 1, 123-132.

Blowers, A. D., Ellmore, G. S., Klein, U., and Bogorad, L. (1990). Transcriptional analysis of endogenous and foreign genes in chloroplast transformants of *Chlamydomonas*. Plant Cell 2, 1059-1070.

Blowers, A. D., Klein, U., Ellmore, G. S., and Bogorad, L. (1993). Functional *in vivo* analyses of the 3' flanking sequences of the *Chlamydomonas* chloroplast *rbcL* and *psaB* genes. Mol Gen Genet 238, 339-349.

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 et al. (1988). Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240, 1534-1538.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.

Braun, F., Le Derout, J., and Regnier, P. (1998). Ribosomes inhibit an RNase E cleavage which induces the decay of the *rpsO* mRNA of *Escherichia coli*. EMBO J 17, 4790-4797.

Bruick, R. K., and Mayfield, S. P. (1998). Processing of the *psbA* 5' untranslated region in *Chlamydomonas reinhardtii* depends upon factors mediating ribosome association. J Cell Biol 143, 1145-1153.

Burkard, G. (1974). Poly(A) polymerase and poly(G) polymerase in wheat chloroplast. Proc. Natl Acad. Sci. USA 71, 389-393.

Choquet, Y., Stern, D. B., Wostrikoff, K., Kuras, R., Girard-Bascou, J., and Wollman, F. A. (1998). Translation of cytochrome f is autoregulated through the 5'-untranslated region of petA mRNA in *Chlamydomonas* chloroplasts. Proc Natl Acad Sci U S A 95, 4380-4385.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA 81, 1991-1995.

Cohen, A., Yohn, C. B., Bruick, R. K., and Mayfield, S. P. (1998). Translational regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. In Methods in enzymology, L. McIntosh, ed. (San Diego: Academic Press), pp. 192-208.

Cohen, S. N. (1995). Surprises at the 3' end of prokaryotic RNA. Cell 80, 829-832.

Cohen, S. N., and McDowall, K. J. (1997). RNase E: still a wonderfully mysterious enzyme. Mol Microbiol 23, 1099-1106.

Delepelaire (1983). Characterization of additional thylakoid membrane polypeptides synthesized inside the chloroplast in *Chlamydomonas reinhardtii*. Photobiochemistry and photobiophysics 6, 279-291.

Deng, X. W., and Gruissem, W. (1987). Control of plastid gene expression during development: the limited role of transcriptional regulation. Cell 49, 379-387.

Deng, X. W., Stern, D. B., Tonkyn, J. C., and Gruissem, W. (1987). Plastid run-on transcription. Application to determine the transcriptional regulation of spinach plastid genes. J Biol Chem 262, 9641-9648.

Drager, R. G., Girard-Bascou, J., Choquet, Y., Kindle, K. L., and Stern, D. B. (1998). *In vivo* evidence for 5'-->3' exoribonuclease degradation of an unstable chloroplast mRNA. Plant J 13, 85-96.

Drapier, D., Girard-Bascou, J., and Wollman, F. A. (1992). Evidence for nuclear control of the expression of the *atpA* and *atpB* chloroplast genes in *Chlamydomonas*. The Plant Cell 4, 283-295.

Dron, M., Rahire, M., and Rochaix, J. D. (1982). Sequence of the chloroplast 16S rRNA gene and its surrounding regions of *Chlamydomonas reinhardii*. Nucleic Acids Res 10, 7609-7620.

Edhofer, I., Muhlbauer, S. K., and Eichacker, L. A. (1998). Light regulates the rate of translation elongation of chloroplast reaction center protein D1. Eur J Biochem 257, 78-84.

Erickson, J. M., Rahire, M., Malone, P., Girard-Bascou, J., and Pierre, Y. (1986). Lack of the D2 protein in Chlamydomonas reinhardtii *psbD* mutant affects photosystem II stability and D1 expression. EMBO J 5, 1745-1754.

Gay, D. A., Sisodia, S. S., and Cleveland, D. W. (1989). Autoregulatory control of β-tubulin mRNA stability is linked to translation elongation. Proc Natl Acad Sci USA 86, 5763-5767.

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

Gong, Z. Y., and Brandhorst, B. P. (1988). Stabilization of tubulin mRNA by inhibition of protein synthesis in sea urchin embryos. Mol Cell Biol 8, 3518-3525.

Gruissem, W., and Schuster, G. (1993). Control of mRNA degradation in organelles, J. Belasco and G. Brawerman, eds. (Orlando: Academic Press).

Gruissem, W., and Tonkyn, J. C. (1993). Control mechanisms of plastid gene expression. Critical reviews in Plant Science 12, 19-55.

Gruissem, W., and Zurawski, G. (1985). Analysis of promoter regions for the spinach chloroplast *rbcL*, *atpB* and *psbA* genes. EMBO J 4, 3375.

Hanson, M. R., Sutton, C. A., and Lu, B. (1996). Plant organelle gene expression: altered by RNA editig. Trends in Plant Science 1, 57-64.

Harris, E. H. (1985). The *Chlamydomonas* sourcebook: a comprehensive guide to biology and laboratory use. (San Diego: Academic Press).

Hauser, C. R., Gillham, N. W., and Boynton, J. E. (1996). Translational regulation of chloroplast genes. Proteins binding to the 5'-untranslated regions of chloroplast mRNAs in *Chlamydomonas reinhardtii*. J Biol Chem 271, 1486-1497.

Hayes, R., Kudla, J., Schuster, G., Gabay, L., Maliga, P., and Gruissem, W. (1996). Chloroplast mRNA 3'-end processing by a high molecular weight protein complex is regulated by nuclear encoded RNA binding proteins. EMBO J 15, 1132-1141.

Higgs, D. C., Esposito, D., Kindle, K. L., and Stern, D. B. (1997). RNA stability and translational regulatory elements in the 5'-untranslated regions of two *Chlamydomonas* chloroplast transcripts. Nucleic Acids Symp Ser 36, 132-134.

Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C. R., Meng, B. Y., and et al. (1989). The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol Gen Genet 217, 185-194.

Hollingshead, S., and Vapnek, D. (1985). Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenylyltransferase. Plasmid 13, 17-30.

Hong, S., and Spreitzer, R. J. (1998). Nuclear-gene mutations suppress a defect in the expression of the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol 116, 1387-1392.

Iost, I., and Dreyfus, M. (1995). The stability of *Escherichia coli lacZ* mRNA depends upon the simultaneity of its synthesis and translation. EMBO J 14, 3252-3261.

Jefferson, R. A., Burgess, S. M., and Hirsh, D. (1986). β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci USA 83, 8447-8451.

Kapoor, S., Suzuki, J. Y., and Sugiura, M. (1997). Identification and functional significance of a new class of non- consensus-type plastid promoters. Plant J 11, 327-337.

Klaff, P., and Gruissem, W. (1991). Changes in chloroplast mRNA stability during leaf development. The Plant Cell 3, 517-529.

Klein, R. R., Mason, H. S., and Mullet, J. E. (1988). Light-regulated translation of chloroplast proteins. I. Transcripts of *psaA-psaB*, *psbA*, and *rbcL* are associated with polysomes in dark-grown and illuminated barley seedlings. J Cell Biol 106, 289-301.

Klein, U., De Camp, J. D., and Bogorad, L. (1992). Two types of chloroplast gene promoters in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 89, 3453-3457.

Klein, U., Salvador, M. L., and Bogorad, L. (1994). Activity of the *Chlamydomonas* chloroplast *rbcL* gene promoter is enhanced by a remote sequence element. Proc Natl Acad Sci USA 91, 10819-10823.

Kuchka, M. R., Goldschmidt-Clermont, M., van Dillewijn, J., and Rochaix, J. D. (1989). Mutation at the *Chlamydomonas* nuclear NAC2 locus specifically affects stability of the chloroplast *psbD* transcript encoding polypeptide D2 of PS II. Cell 58, 869-876.

Kudla, J., Hayes, R., and Gruissem, W. (1996). Polyadenylation accelerates degradation of chloroplast mRNA. EMBO J 15, 7137-7146.

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

Lagunez-Otero, J. (1993). rRNA-mRNA complementarity: implications for translation initiation. Trends Biochem Sci 18, 406-408.

Laird-Offringa, I. A., de Wit, C. L., Elfferich, P., and van der Eb, A. J. (1990). Poly(A) tail shortening is the translation-dependent step in *c-myc* mRNA degradation. Mol Cell Biol 10, 6132-6140.

Li, Y. Q., and Sugiura, M. (1990). Three distinct ribonucleoproteins from tobacco chloroplasts: each contains a unique amino terminal acidic domain and two ribonucleoprotein consensus motifs. EMBO J 9, 3059-3066.

Linial, M., Gunderson, N., and Groudine, M. (1985). Enhanced transcription of *c-myc* in bursal lymphoma cells requires continuous protein synthesis. Science 230, 1126-1132.

Lisitsky, I., Klaff, P., and Schuster, G. (1996). Addition of destabilizing poly (A)-rich sequences to endonuclease cleavage sites during the degradation of chloroplast mRNA. Proc Natl Acad Sci USA 93, 13398-13403.

Lisitsky, I., Kotler, A., and Schuster, G. (1997). The mechanism of preferential degradation of polyadenylated RNA in the chloroplast. The exoribonuclease 100RNP/polynucleotide phosphorylase displays high binding affinity for poly(A) sequence. J Biol Chem 272, 17648-17653.

Maier, R. M., Neckermann, K., Igloi, G. L., and Kossel, H. (1995). Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. J Mol Biol 251, 614-628.

Maliga, P., and Nixon, P. J. (1998). Judging the homoplastomic state of plastid transformation. Trends in Plant Science 3, 376.

Manley, J. L. (1995). Messenger RNA polyadenylation: A universal modification. Proc. Natl. Acad. Sci. USA 92, 1800-1801.

Mayfield, S. P., Yohn, C. B., Cohen, A., and Danon, A. (1995). Regulation of chloroplast gene expression. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 147-166.

McCarthy, J. E., and Brimacombe, R. (1994). Prokaryotic translation: the interactive pathway leading to initiation. Trends Genet 10, 402-407.

Mullet, J. E. (1988). Chloroplast development and gene expression. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 475-502.

Nickelsen, J., van Dillewijn, J., Rahire, M., and Rochaix, J. D. (1994). Determinants for stability of the chloroplast *psbD* RNA are located within its short leader region in *Chlamydomonas reinhardtii*. EMBO J 13, 3182-3191.

Nilsson, G., Belasco, J. G., Cohen, S. N., and von Gabain, A. (1987). Effect of premature termination of translation on mRNA stability depends on the site of ribosome release. Proc Natl Acad Sci USA 84, 4890-4894.

O'Hara, E. B., Chekanova, J. A., Ingle, C. A., Kushner, Z. R., Peters, E., and Kushner, S. R. (1995). Polyadenylylation helps regulate mRNA decay in *Escherichia coli* . Proc Natl Acad Sci USA 92, 1807-1811.

Ohyama K, F. H., Kohchi T, Sirai H, Sano T, et al (1986). Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322, 572-574.

Parker, R., and Jacobson, A. (1990). Translation and a 42-nucleotide segment within the coding region of the mRNA encoded by the MAT alpha 1 gene are involved in promoting rapid mRNA decay in yeast. Proc Natl Acad Sci USA 87, 2780-2784.

Petersen, C. (1993). Translation and mRNA stability in bacteria: a complex relationship, J. Belasco and G. Brawerman, eds. (Orlando: Academic Press).

Piechulla, B., and Gruissem, W. (1987). Diurnal mRNA fluctuations of nuclear and plastid genes in developing tomato fruits. EMBO J 6, 3593-3599.

Rochaix, J. D. (1996). Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. Plant Molecular Biology 32, 327-341.

Rochaix, J. D. (1992). Post-transcriptional steps in the expression of chloroplast genes. Annu. Rev. Cell Biol. 8, 1-28.

Rochaix, J. D., Kuchka, M., Mayfield, S., Schirmer-Rahire, M., Girard-Bascou, J., and Bennoun, P. (1989). Nuclear and chloroplast mutations affect the synthesis or stability of the chloroplast *psbC* gene product in *Chlamydomonas reinhardtii*. EMBO J 8, 1013-1021.

Rochaix, J. D., Mayfield, S., Goldschmidt-Clermont, M., and Erickson, J. (1988). Molecular biology of *Chlamydomonas*, C. H. Shaw, ed. (Oxford: IRL Press).

Rott, R., Drager, R. G., Stern, D. B., and Schuster, G. (1996). The 3' untranslated regions of chloroplast genes in *Chlamydomonas reinhardtii* do not serve as efficient transcriptional terminators. Mol Gen Genet 252, 676-683.

Rott, R., Levy, H., Drager, R. G., Stern, D. B., and Schuster, G. (1998a). 3'-Processed mRNA is preferentially translated *in Chlamydomonas reinhardtii* chloroplasts. Mol Cell Biol 18, 4605-4611.

Rott, R., Liveanu, V., Drager, R. G., Stern, D. B., and Schuster, G. (1998b). The sequence and structure of the 3'-untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability. Plant Mol Biol 36, 307-314.

Sakamoto, W., Chen, X., Kindle, K. L., and Stern, D. B. (1994). Function of *the Chlamydomonas reinhardtii petD* 5'-untranslated region in regulating the accumulation of subunit IV of the cytochrome b6/f complex. Plant J 6, 503-512.

Sakamoto, W., Kindle, K. L., and Stern, D. B. (1993). *In vivo* analysis of *Chlamydomonas chloroplast petD* gene expression using stable transformation of beta-glucuronidase translational fusions. Proc Natl Acad Sci USA 90, 497-501.

Sakamoto, W., Sturm, N. R., Kindle, K. L., and Stern, D. B. (1994). *petD* mRNA maturation in *Chlamydomonas reinhardtii* chloroplasts: role of 5' endonucleolytic processing. Mol Cell Biol 14, 6180-6186.

Salvador, M. L., Klein, U., and Bogorad, L. (1993). 5' sequences are important positive and negative determinants of the longevity of *Chlamydomonas* chloroplast gene transcripts. Proc Natl Acad Sci USA 90, 1556-1560.

Salvador, M. L., Klein, U., and Bogorad, L. (1993). Light-regulated and endogenous fluctuations of chloroplast transcript levels in *Chlamydomonas*. Regulation by transcription and RNA degradation. Plant J 3, 213-219.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning Second edition (New York: Cold Spring Harbor Laboratory Press).

Schuster, G., and Gruissem, W. (1991). Chloroplast mRNA 3' end processing requires a nuclear-encoded RNA- binding protein. EMBO J 10, 1493-1502.

Sexton, T. B., Christopher, D. A., and Mullet, J. E. (1990). Light-induced switch in barley *psbD-psbC* promoter utilization: a novel mechanism regulating chloroplast gene expression. EMBO J 9, 4485-4494.

Shapira, M., Lers, A., Heifetz, P. B., Irihimovitz, V., Osmond, C. B., Gillham, N. W., and Boynton, J. E. (1997). Differential regulation of chloroplast gene expression in *Chlamydomonas reinhardtii* during photoacclimation: light stress transiently suppresses synthesis of the Rubisco LSU protein while enhancing synthesis of the PS II D1 protein. Plant Mol Biol 33, 1001-1011.

Shiina, T., Allison, L., and Maliga, P. (1998). *rbcL* Transcript levels in tobacco plastids are independent of light: reduced dark transcription rate is compensated by increased mRNA stability. Plant Cell 10, 1713-1722.

Shinozaki K, O. M., Tanaka M, Wakasugi T, Hayashida N, et al (1986). The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J. 5, 2043-2049.

Spreitzer, R. J., Goldschmidt-Clermont, M., Rahire, M., and Rochaix, J. D. (1985). Nonsense mutations in the *Chlamydomonas* chloroplast gene that codes for the large subunit of ribulosebisphosphate carboxylase/oxygenase. Proc. Natl. Acad. Sci. USA 82, 5460-5464.

Stampacchia, O., Girard-Bascou, J., Zanasco, J. L., Zerges, W., Bennoun, P., and Rochaix, J. D. (1997). A nuclear-encoded function essential for translation of the chloroplast *psaB* mRNA in *Chlamydomonas*. Plant Cell 9, 773-782.

Stern, D. B., and Gruissem, W. (1987). Control of plastid gene expression: 3' inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. Cell 51, 1145-1157.

Stern, D. B., Jones, H., and Gruissem, W. (1989). Function of plastid mRNA 3' inverted repeats. RNA stabilization and gene-specific protein binding. J Biol Chem 264, 18742-18750.

Stern, D. B., Radwanski, E. R., and Kindle, K. L. (1991). A 3' stem/loop structure of the *Chlamydomonas* chloroplast *atpB* gene regulates mRNA accumulation in vivo. Plant Cell 3, 285-297.

Stimac, E., Groppi, V. E., Jr., and Coffino, P. (1984). Inhibition of protein synthesis stabilizes histone mRNA. Mol Cell Biol 4, 2082-2090.

Sugita, M., and Sugiura, M. (1996). Regulation of gene expression in chloroplasts of higher plants. Plant Mol Biol 32, 315-326.

Sugiura, M. (1992). The chloroplast genome. Plant Mol Biol 19, 149-168.

Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990). Stable transformation of plastids in higher plants. Proc. Natl. Acad. Sci. USA 87, 8526-8530.

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.

Tanaka, K., Oikawa, K., Ohta, N., Kuroiwa, H., Kuroiwa, T., and Takahashi, H. (1996). Nuclear encoding of a chloroplast RNA polymerase sigma subunit in a red alga. Science 272, 1932-1935.

Theodorakis, N., and Cleveland, D. (1996). Translationally coupled degradation of mRNA in eukaryoted, J. W. B. Hershey, M. B. Mathews and N. Sonenberg, eds. (New York: Cold Spring Harbor Laboratory Press).

To, K. Y., Cheng, M. C., Suen, D. F., Mon, D. P., Chen, L. F., and Chen, S. C. (1996). Characterization of the light-responsive promoter of rice chloroplast *psbD-C* operon and the sequence-specific DNA binding factor. Plant Cell Physiol 37, 660-666.

Troxler, R. F., Zhang, F., Hu, J., and Bogorad, L. (1994). Evidence that sigma factors are components of chloroplast RNA polymerase. Plant Physiol 104, 753-759.

Vazquez, D. (1979). Inhibitors of protein biosynthesis. Mol Biol Biochem Biophys 30, 1-312.

Wakasugi, T., Nagai, T., Kapoor, M., Sugita, M., Ito, M., Ito, S., Tsudzuki, J., Nakashima, K., Tsudzuki, T., Suzuki, Y., Hamada, A., Ohta, T., Inamura, A., Yoshinaga, K., and Sugiura, M. (1997). Complete nucleotide sequence of the chloroplast genome from the green alga *Chlorella vulgaris*: the existence of genes possibly involved in chloroplast division. Proc Natl Acad Sci USA 94, 5967-5972.

Wilson, T., and Treisman, R. (1988). Fos C-terminal mutations block down-regulation of *c-fos* transcription following serum stimulation. EMBO J 7, 4193-4202.

Xu, R., Bingham, S. E., and Webber, A. N. (1993). Increased mRNA accumulation in a *psaB* frame-shift mutant of *Chlamydomonas reinhardtii* suggests a role for translation in *psaB* mRNA stability. Plant Mol Biol 22, 465-474.

Ye, L., and Sugiura, M. (1992). Domains required for nucleic acid binding activities in chloroplast ribonucleoproteins. Nucleic Acids Res 20, 6275-6279.

Ye, L. H., Li, Y. Q., Fukami-Kobayashi, K., Go, M., Konishi, T., Watanabe, A., and Sugiura, M. (1991). Diversity of a ribonucleoprotein family in tobacco chloroplasts: two new chloroplast ribonucleoproteins and a phylogenetic tree of ten chloroplast RNA-binding domains. Nucleic Acids Res 19, 6485-6490.

Zerges, W., Girard-Bascou, J., and Rochaix, J. D. (1997). Translation of the chloroplast *psbC* mRNA is controlled by interactions between its 5'-leader and the nuclear loci TBC1 and TBC3 in *Chlamydomonas reinhardtii*. Mol Cell Biol 17, 3440-3448.

Zerges, W., and Rochaix, J. D. (1994). The 5'-leader of a chloroplast mRNA mediates the translational requirements for two nucleus-encoded functions in *Chlamydomonas reinhardtii*. Mol Cell Biol 14, 5268-5277.

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