Point mutations in the 5´UTR of the *Chlamydomonas reinhardtii rbcL* gene destabilize its transcript

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Cand. Scient thesis in biotechnology
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University of Oslo, August 2004

ABSTRACT: A reporter gene was constructed consisting of the 5´ region of the *C. reinhardtii rbcL* gene from position –70 to +47, relative to the transcription start point, fused to the *E. coli uidA*(GUS) structural gene terminated by the *Chlamydomonas psaB 3´* end. Two point mutations were introduced into this construct at positions +46 and +47 (A→T and C→T, respectively) in the *rbcL* 5´UTR. The construct was introduced into the *Chlamydomonas* chloroplast genome downstream of the *atpB* gene. Transcript accumulation from this reporter gene was determined by a Northern blot assay. It was found that GUS transcripts did not accumulate.

Acknowledgments:

This work was carried out at the University of Oslo (UiO), Department of Molecular Bioscience (MBV), under the supervision of Professor Uwe Klein.
I would like to thank Professor Klein for his help and guidance both in the laboratory and in the writing of this thesis.
I would also like to thank my fellow students for providing a stimulating work environment. Especially I would like to thank Pål Trosvik for critically reading the first draft of the thesis.

CONTENTS

1. INTRODUCTION

The plastids, among them the chloroplast, are generally believed to have originated from an endosymbiosis between an eukaryotic host and a photosynthetic cyanobacterium. Recent phylogenetic analysis suggests that chloroplasts in land plants, green algae, red algae, and glaucophytes all originate from a single, ancient, endosymbiotic event (Moreira *et al.* 2000). Also, it has been found that the transcription and translation apparatuses in the chloroplast are basically of a prokaryotic nature. In the course of evolution the chloroplast has adopted several mechanisms for controlling RNA and protein synthesis. The current view is that expression of many chloroplast-encoded genes is regulated by nuclear encoded factors that act mainly at the posttranscriptional level (Nickelsen 2003).
This study looked at how a deletion in the 5´untranslated region (5´UTR) of the chloroplast encoded *rbcL* gene of the green alga *Chlamydomonas reinhardtii* (hereafter referred to as *Chlamydomonas*) affects the *in vivo* accumulation of transcripts of a reporter gene construct consisting of the *rbcL* promoter and 5´UTR from *Chlamydomonas* fused to the *E. coli uidA* (β-glucuronidase; GUS) gene.

1.1 Promoter types and RNA-polymerases in the chloroplast.

Plastid genes in higher plants have been found to be transcribed by two different types of polymerases: a plastid encoded polymerase (PEP) and a nuclear encoded polymerase (NEP). The PEP which is homologous to the *E. coli* RNA polymerase, consisting of subunits α, β, β´, and β´´ encoded by the plastid genes *rpoA*, *rpoB*, and *rpoC*, transcribes genes from *E. coli* σ^{70} -like promoters with –35 and –10 promoter elements (consensus: - 35 TTGACA, –10 TATAAT). *rpoC* is often divided into two distinct transcription units; *rpoC1* and *rpoC2*. The PEP is dependent upon nuclear encoded σ-like factors, offering the nucleus some control over gene expression in the chloroplast (Hajdukiewicz *et al.* 1997, Kung and Lin 1985). It has been suggested that more than one PEP exists in some plants. In this model, the catalytic core of the polymerase remains the same, but accessory factors (other than the σ-like factors) vary with developmental stages (Cahoon and Stern 2001). The genes encoding the PEP of *Chlamydomonas* are unusual in that no *rpoC1* homologue has been found in the chloroplast. Instead a single *rpoC2* gene has been identified. This gene shares homology with other *rpoC2* genes, but also has unique sequences flanking the homologous region. In addition, the *rpoB* gene seems to be divided into two separate transcription units (Maul *et al.* 2002).
When *rpo* genes in the chloroplast are disrupted, transcription of chloroplast genes can still be detected in most plants. These genes are transcribed by a nuclear encoded polymerase (NEP). Cloning and characterization of this polymerase has revealed that it is a single subunit (with respect to the catalytic core) polymerase homologous to the T7/T3 phage polymerases. The promoter for this polymerase consists of a conserved YRT motif near the transcription start

point, and often a conserved 10 nt sequence around the transcription start site (Magee and Kavanagh 2002). There is experimental data suggesting that *Chlamydomonas* lacks a NEP type RNA polymerase, since attempts to delete *rpo* genes have produced mutants in which transcription of chloroplast genes cannot be detected (Lilly *et al.* 2002).

In addition to the above-mentioned promoters, genes with internal promoters have also been described. These genes are not dependent upon transcription-promoting sequences upstream of the gene, but rather depend on sequences within the transcribed region to promote transcription. The polymerase responsible for transcribing these genes has not been characterized. Finally, many genes, like the tobacco *atpB* gene, have both PEP and NEP type promoters driving their transcription.

NEP dependent transcription is typically activated early in plastid development, driving the expression of "housekeeping genes"/ "genetic system" genes like the *rpo* genes and genes involved in the plastid translational system. PEP dependent genes, on the other hand, are typically activated later in plastid development, and encompass genes involved in photosynthesis (Stern *et al.* 1997).

Sequence specific DNA binding factors that are required for effective transcription from plastid promoters have also been described. For instance, Kim and Mullet (1995) described a factor termed AGF in barley that bound to a sequence upstream of the *psbD-psbC* promoter, and that was necessary for transcription to occur from this promoter.

The lack of a NEP in *Chlamydomonas* would suggest that the promoters in the chloroplast should be of the bacterial type, and indeed a σ^{70} type promoter with –35 and -10 elements has been described, e.g. for the 16S rRNA gene. However, in the same study the *atpB* promoter was found to lack a functional –35 element, while retaining an extended –10 sequence (TATAATAT). This promoter also required sequences in the area from approximately 22 bp upstream to approximately 60 bp downstream of the transcription initiation site for maximum transcription (Klein *et al.* 1991). Sakamoto *et al,* (1992) showed that the *petD* gene has a conserved –10 element, but the study was not able to demonstrate the existence of a conserved –35 element for this gene. This would suggest that the lack of a –35 consensus sequence is not entirely uncommon for *Chlamydomonas* chloroplast genes.

An internal type promoter, described for higher plant chloroplasts in the text above, has also been found in the *Chlamydomonas* chloroplast for the tRNA (Glu) gene (Jahn 1992).

The promoter for the gene investigated in this study, the *Chlamydomonas rbcL* gene, has been characterized extensively. Initially the *rbcL* promoter was reported to be contained within the –18 to +63 region relative to the transcription start point, and to lack a conserved –35 element, like the *atpB* promoter (Klein *et al.* 1994). However, a more recent study showed that a –10 TATAATAT (second T at –10) element by itself was able to drive transcription of the *rbcL* gene, and that the reduction in mRNA accumulation associated with changes in the region downstream to nucleotide +63 was caused by a reduction in RNA stability rather than by a reduced rate of transcription (Salvador *et al.* 2004). The –10 element

alone is only able to sustain a basal level of transcription. A *cis*-acting enhancer-like sequence located in the 5´ transcribed region between position +108 and +143 is necessary for full rates of transcription of the *rbcL* gene (Anthonisen *et al.* 2002).

1.2 RNA stability and posttranscriptional regulation of chloroplast gene expression.

As mentioned above, much of the control of chloroplast gene expression takes place at the posttranscriptional level, and is mediated by nuclear factors. Several nuclear mutations that affect the stability of chloroplast transcripts have been described. Examples of such mutations are the nuclear mutants 6.2z5 and GE2.10, described for *Chlamydomonas,* which destabilize the products of the *psbB* and *psbC* genes, respectively. The mutations were shown to reduce the half-life of the mRNA of these genes, but not their rate of transcription (Sieburth *et al.* 1991). Similar mutations have also been described for several other genes in the *Chlamydomona* chloroplast (Drapier *et al.* 1992, Esposito *et al.* 2000). These mutations seem to act in a gene-specific manner, with one nuclear mutation affecting the stability of one or a few chloroplast transcripts, without affecting other transcripts. This might be a difference between *Chlamydomonas* and higher plants, where related mutations more often seem to influence the stability of a greater number of transcripts. For example, the hcf109 nuclear mutation in *Arabidopsis* reduces the stability of transcripts from the *psbB, psbD/C, ndhH,* and *ndhC* operons (Meurer *et al.* 1996).
Cis-acting sequences in the RNAs of chloroplast-encoded genes are important in determining the longevity of their transcripts (Salvador *et al.* 1993b). These sequences have been found both in the 5´UTR and in the coding region, as exemplified by the *Chlamydomonas petD* and *atpA* genes, respectively (Sakamoto *et al.* 1993, Drapier *et al.* 2002). Sequences in the transcripts of higher plants that play a role in transcript longevity have been discovered as well. In tobacco, for example, a stem-loop structure in the 5´UTR is vital for protecting the *psbA* transcript from degradation (Zou *et al*. 2003). Presumably, the factors encoded by the genes disrupted by the above mentioned nuclear mutations directly or indirectly interact with these *cis*-acting sequences, and thereby impede RNA degradation. One such factor, the product of the *Chlamydomonas Nac2* gene, has been cloned and characterized. This gene encodes a 140 kDa protein that was shown to be localized in the chloroplast stroma, and to stabilize the *psbD* mRNA. It was also shown that the Nac2 protein is part of multiprotein complex that probably interacted with the *psbD*-5´UTR (Boudreau *et al.* 2000). Similarly, the product of the cloned *Chlamydomonas* nuclear gene *Mbb1* was able to restore *psbB*-mRNA levels in nuclear mutants that did not accumulate this gene's transcript. Mbb1 was shown to localize to the chloroplast stroma, but it was not demonstrated that this protein associated with *psbB*-mRNA (Vaistij *et al.* 2000). Stem-loop structures in the 3´ends of chloroplast RNAs have also been proposed to play a role in protecting transcripts from degradation. The mechanism of this stabilization by 3´UTRs is not well

defined, but it has been suggested that the structure in the RNA in itself is not enough to account for this stabilizing effect. Instead it has been claimed that factors that bind specifically to the 3´ends are necessary (Stern *et al*. 1993). Specific sequences and secondary structures in the 5´UTR of the mRNA of the *Chlamydomonas rbcL* gene have been implicated in protecting it from degradation.

This comprises a stem-loop made up of sequences in the first 41 nt of the UTR, and the sequence AUUUCCGGA extending from position +38 to + 46 relative to the transcription start point. Changing the sequence in the first 41 nt of the UTR does not influence RNA stability as long as the secondary structure of the stem-loop and the sequence between position +38 and +46 is unchanged (Anthonisen *et al.* 2001, Salvador *et al.* 2004, Uwe Klein, personal communication). These findings all come from studies carried out on reporter gene constructs made up of the *rbcL* promoter and 5´UTR fused to the GUS reporter gene. Earlier reports have shown that reporter gene constructs harboring the *rbcL* 5´UTR are rapidly degraded upon illumination (Salvador *et al*. 1993a). In contrast to this, the endogenous *rbcL* gene transcript has been found to be relatively stable both in light and dark-grown cells, although the stability decreased somewhat in the light. However, this was compensated by increased transcription rates in light-grown cells, so that transcript levels remained at a relatively steady state (Salvador *et al.* 1993b). Interestingly, a 2001 study by Singh *et al.* found that a sequence between position +21 and +41 is implicated in the photo-accelerated degradation of transcripts containing the *rbcL* 5´UTR. This sequence lies within the same stem-loop structure that seems to confer a general stabilizing effect on *rbcL* 5´UTR containing transcripts. A stem-loop structure in the coding region of the gene, around position +327, was found to counter the destabilizing effect of the +21 to +41 sequence. The loop portion of this stem-loop was hypothesized to interact, directly or indirectly, with the loop of the previously described stem-loop in the first 41 nt of the 5´UTR, and thereby restoring, at least to some extent, the relative stability found for the endogenous *rbcL* gene in the light. The factor(s) responsible for bestowing the light regulation on the *rbcL* gene is not known. Nevertheless, Salvador *et al*. (1999) demonstrated that this factor(s) is regulated by the redox state of the chloroplast. It was shown that the degradation in light was dependent upon the reducing potential that is created by the photosynthetic electron flow.

Studies on the 3´UTR of the gene have revealed that the 3´end probably is not important in determining the stability of the RNA, but that it plays a role in end maturation (Blowers *et al.* 1993).

The details of the machinery that is responsible for RNA degradation in the chloroplast have not been defined to date, although some of the factors involved have been delineated. These include both 3´→5´ exoribonucleases and endoribonucleases. The possible existence of a 5´→3´ exoribonuclease in the chloroplast of *Chlamydomonas* was first inferred from studies on *petD*-mRNA. A nuclear mutant that had previously been shown to lack a factor that stabilized the *petD*-mRNA by interacting with its 5´UTR was able to accumulate a *petD*-reporter construct when a poly-G sequence was fused upstream of the *petD*-

5´UTR. Poly-G sequences are known to impede exoribonucleases, indicating that a 5´→3´exoribonuclease is involved in RNA degradation in *Chlamydomonas* chloroplasts (Drager *et al*. 1998). Although this does not prove the existence of a 5´→3´ exoribonuclease in the chloroplast of *Chlamydomonas* it has been proposed that the role of a 5´→3´ nuclease in plastid RNA degradation is unique to *Chlamydomonas,* since similar factors with 5´→3´exoribonuclease activity have not been found in plastids from higher plants to date.

Currently, the only 3´→5´exoribonuclease that has been identified in chloroplasts is the polynucleotide phosphorylase (PNPase). PNPase has been implicated in both 3´end maturation and in RNA turnover (Walter *et al.* 2002). Endoribonucleases have been found in the chloroplasts of several species of photosynthetic organisms, e.g. CSP41 from spinach, which preferentially cuts RNA in stem-loop structures, and a 54kDa enzyme from mustard proposed to play a role in 3´end maturation (Monde *et al.* 2000). Presently, polyadenylation is thought to have an important role in RNA degradation by PNPase. In this model degradation is initiated by an endoribonucleolytic cut of the RNA upstream of the 3´end, which contains sequences/structures that protect the transcript from degradation by PNPase. The free 3´ends are polyadenylated by an as of now unidentified enzyme, and the polyadenylated RNAs are quickly degraded by PNPase. According to the model it is possible that more than one cut is made in the RNA by endoribonucleases, creating many small polyadenylated molecules (Schuster *et al.* 1999).

In addition to the processes involved in RNA turnover discussed above, post-transcriptional modifications such as processing of polycistronic transcripts, intron splicing, and RNA editing take place in the chloroplast (Reviewed in Monde *et al.* 2000).

Finally, it has been demonstrated that several chloroplast genes are regulated at the translational level in a process termed control by epistasis of synthesis (CES). In this process, the translation of certain proteins that are part of multi-subunit complexes are controlled by the presence of its assembly partners. For example, the *petA*-mRNA of *Chlamydomonas*, encoding cytochrome *f* of the cytochrome *bf* complex, is not translated in the absence of the subunit IV protein of this complex. This attenuation of translation occurs because the C-terminal end of the unassembled cytochrome *f* protein interacts with, and sequesters, the translation factor TCAI, which is necessary for *petA*-mRNA translation. When the cytochrome *f* protein is assembled in the normal manner its C-terminal end is not free to interact with TCAI, and subsequently TCAI is available to promote translation of the *petA*-mRNA.

There is evidence to support the theory that the *rbcL* gene of tobacco is regulated in this manner, and it has also been proposed that this is the case for the *Chlamydomons rbcL* gene (Choquet *et al.* 2001).

1.3 *Chlamydomonas reinhardtii*

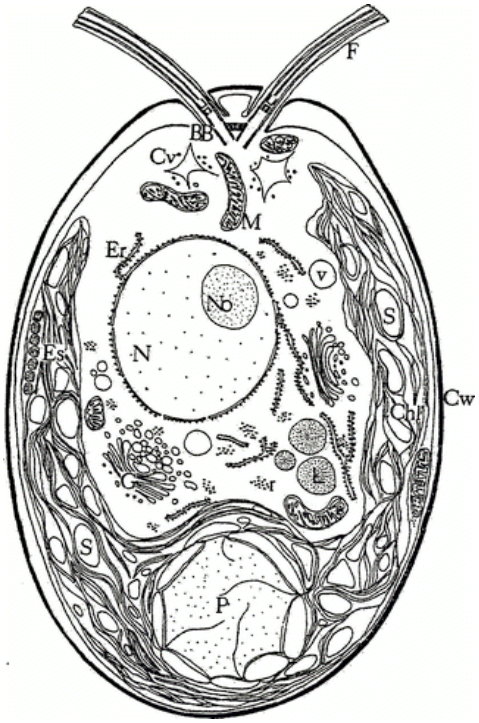


Figure 1 Schematic drawing of *Chlamydomonas reinhardtii*.

BB: basal bodies Chl: chloroplast Cv: contractile vacuole Cw: cell wall Er: endoplasmic reticulum Es: eyespot F: flagella G: Golgi L: lipid body Mi: mitochondria N: nucleus No: nucleolus P: pyrenoid r: ribosomes S: starch grain v: vacuole (Harris 2001).

Chlamydomonas reinhardtii is a unicellular photosynthetic green alga belonging to the genus *Chlamydomonas.* It has a diameter of about 10 μm (depending on the stage of the cell cycle) and two flagella protruding from the anterior of the cell. The flagella are approximately 10-12 μm long, and are used to propel the cell forward with a breaststroke-like motion. A seven-layered cell wall, composed primarily of hydroxyproline-rich glygoproteins, surround the cell. The nucleus contains a ~$1x10^8$ bp genome divided on (probably) 17 chromosomes, and runs continuously with the endoplasmic reticulum. One to four Golgi bodies surround the nucleus. A bright orange eyespot (stigma) can be seen under the light microscope at the equator of the cell. Electron microscopy has revealed that the stigma is located just under the outer membrane of the chloroplast, and that it probably functions in directing light to the retinal binding rhodopsin photoreceptor located in the plasma membrane. The photoreceptor enables the cell to detect unidirectional light, and aids in the phototaxis of the cell. Two contractile vacuoles in the anterior maintain the water balance of the cell (Harris 2001).

1.3.1 The chloroplast, photosynthesis, and rubisco.

The only plastid in *Chlamydomonas* is a single, large, cup-shaped chloroplast that partially surrounds the nucleus. Within the chloroplast a relatively large structure called the pyrenoid can bee seen. The pyrenoid is usually surrounded by starch bodies. Although the exact function of the pyrenoid is uncertain, it is known to contain large amounts of the CO_2 fixing enzyme rubisco (Harris 2001). Chloroplasts are the site of photosynthesis in all photosynthetic eukaryotes. In general, the chloroplast is surrounded by a highly permeable outer membrane and an impermeable inner membrane, which are separated by the intermembrane

space. The inner membrane encloses the stroma. Stacks of flattened membrane sacks, called grana, can be found in the stroma. Each sack in the grana is called a thylakoid. The thylakoids enfold the thylakoid compartment. Stromal lamellae connect the grana, and all grana in a chloroplast are believed to be made up of one highly folded vesicle. Proteins and pigments associated with the thylakoid membrane are responsible for the light reactions of photosynthesis, and the dark reactions take place in the stroma.

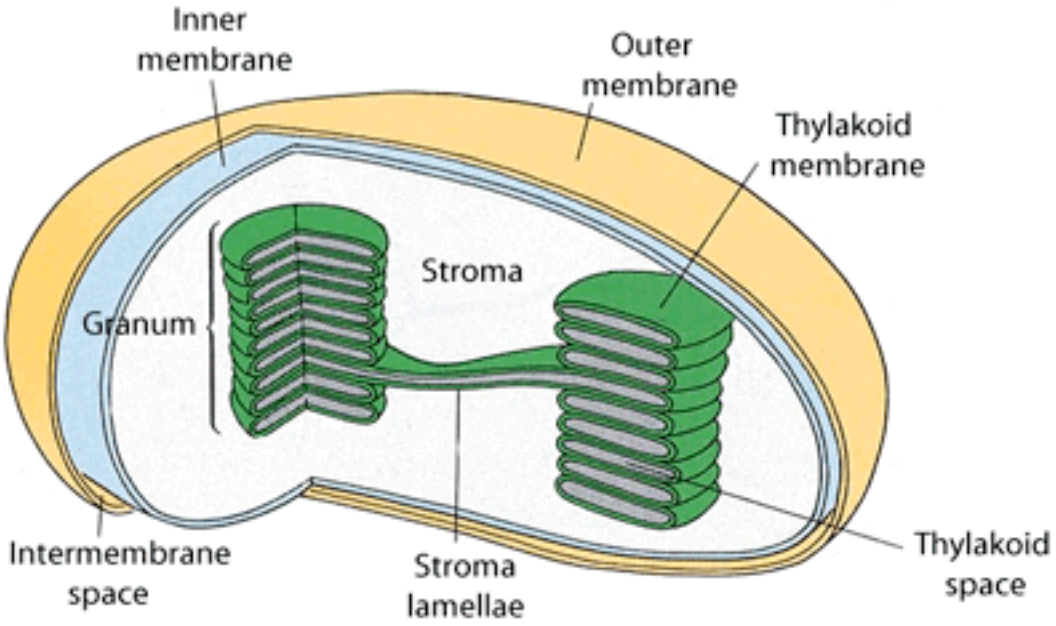


Figure 2 Schematic drawing of a chloroplast.

Source:http://138.192.68.68/bio/Courses/biochem2/Photosynthesis/PhotosynthesisResources/Chloroplast.gif

The product of the gene investigated in this study, the plastid-encoded *rbcL* gene, plays a prominent role in the dark reactions of photosynthesis. Together with the nuclear encoded *rbcS* gene product it makes up the enzyme ribulose-1,5-bisphosphate carboxylase (also known as Rubisco) that catalyzes the reaction between ribulose-1,5-bisphosphate and CO_2. The enzyme is a hetero-hexadecamer consisting of eight large (rbcL) and eight small (rbcS) subunits. The catalytic activity of the enzyme resides in the large subunit. What function the small subunit has is uncertain (Voet and Voet 1995). The proposed mechanism of the enzyme can be seen in the figure below (fig. 3).

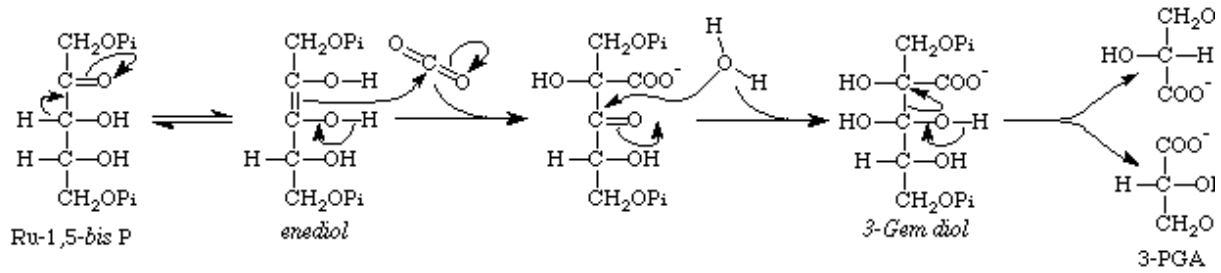


Figure 3 Catalytic mechanism of Rubisco

First the enzyme deprotonates the third carbon of ribulose-1,5-bisphosphate. The resulting deprotonated ribulose-1,5-bisphosphate forms an equilibrium with an enediol intermediate. Electrons from the double bond between carbon 2 and 3 in the enediol nucleophilically attacks the carbon in CO_2 yielding a ß-keto acid intermediate. Oxygen from water attacks carbon three in the ß-keto acid, resulting in the formation of a 3-gem diol. This intermediate is split into two molecules of 3-phosphoglycerate.
Source:*http://www.humboldt.edu/~rap1/C431.F01/C431Notes/ETS_Photo/RuBisCo.gif*

1.3.2 The chloroplast genome

The entire 203,395 bp chloroplast genome of *Chlamydomonas* (fig. 4) has been sequenced, and analysis of the sequence has revealed that the genome is organized into two single copy regions of approximately 80 kb separated by two 21,5 kb inverted repeats. Furthermore, the genome seems to be relatively gene-poor compared with other chloroplast genomes sequenced to date. Genes can be found both in the single copy and inverted repeat regions. Identified genes encompass genes encoding components of the translation system, including tRNAs, rRNAs, and ribosomal proteins, and genes involved in photosynthesis such as photosystem/electron transport chain genes, ATPase genes, and genes concerned with chlorophyll biosynthesis. In addition, several previously unidentified open reading frames of unknown function were uncovered. The genes encoding the PEP were found to be unusual relative to those found in the chloroplast of other photosynthetic organisms (for details on the *Chlamydomonas* PEP see section 1.1).
A feature that separates the *Chlamydomonas* plastid genome from those of other single celled photosynthetic organisms and higher plants sequenced to date is the prevalence of repeated sequences. Some ~20% of the genome seem to be made up of repeated sequences, and numerous families of short dispersed repeats have been discovered throughout the genome.
In vivo the genome exists as both a linear and a circular molecule predominantly as monomers or dimers. It has been estimated that the copy number of the genome in each chloroplast is in the range of 50-80 molecules, depending on growth conditions (Maul *et al.* 2002, Rochaix and Malnoe 1978). The entire chloroplast sequence can be found at the *Chlamydomonas* Genetic Center homepage (http://www.biology.duke.edu/chlamy/index.html).

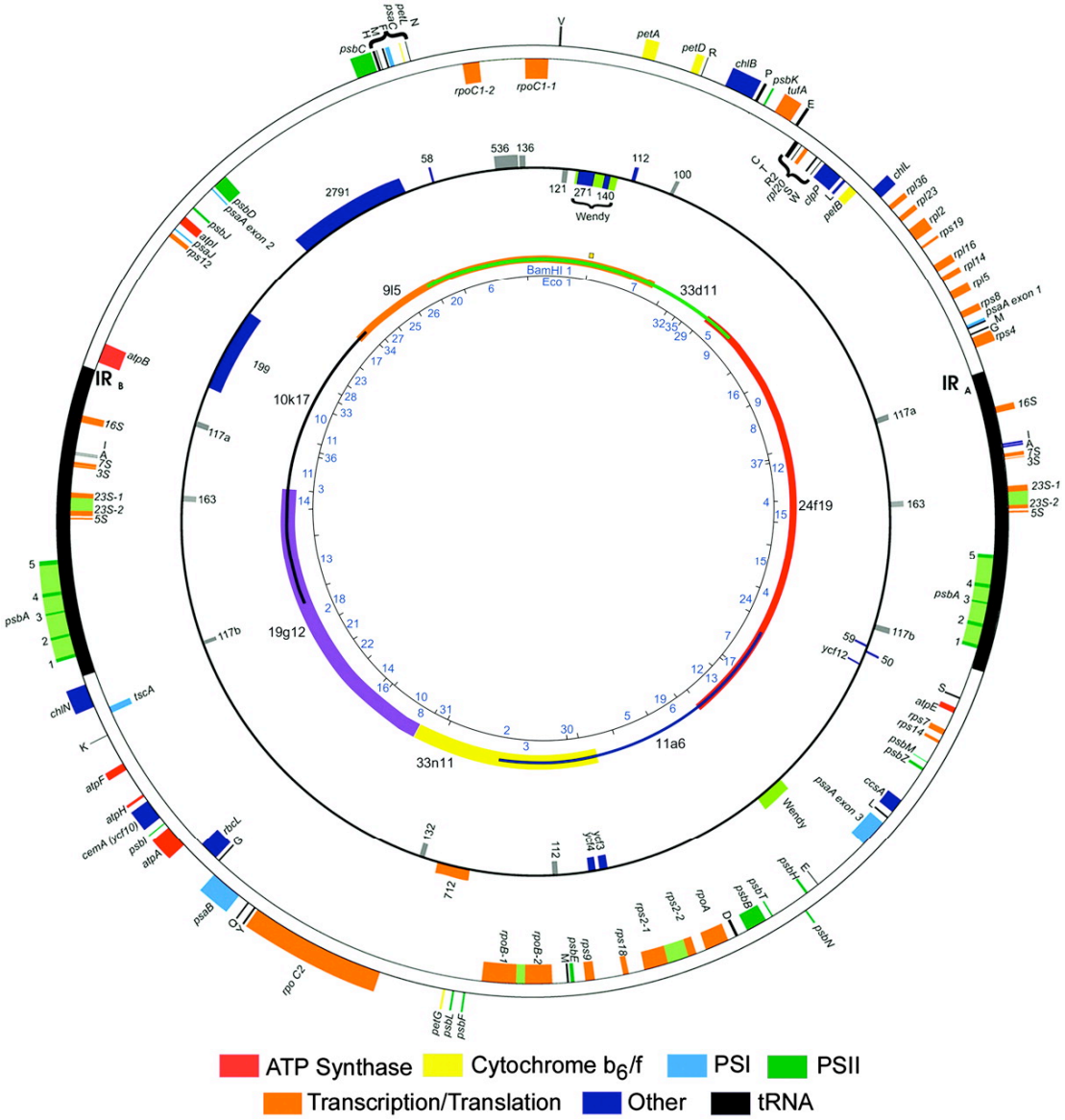


Figure 4 Map of the *Chlamydomonas reinhardtii* chloroplast genome

The inner circle shows the *Bam*HI/*Eco*RI restriction fragment map established by Rochaix (1980) and numbering established by Grant *et al.* (1980). The second circle specifies the seven overlapping BAC clones used in the sequencing of the genome. Circle three represents ORFs and genes of unknown function. The outer circle shows genes of known or presumed function. Genes are color-coded according to gene class. Introns are represented by olive green. Taken from Maul *et al.* (2002).

1.3.3 Life cycle

Wild type cells are normally haploids of two mating types, designated mt+ and mt- that reproduce asexually by mitosis. Upon nitrogen starvation haploid cells enter gametogenesis, and mt+/- gametes are formed. Mating is initiated by pairing of the gametes of the two mating types along their flagella. This is aided by the expression of sex specific agglutinins on the flagella. Pairing is followed by the release of a lytic enzyme that breaks down the cell wall. Fusion of the gametes is initiated at the anterior of the cells by sex specific structures, and continues laterally along the anterior-posterior axis. This leads to the formation of a diploid, quadriflagellat, zygote that remains motile for several hours. Eventually the zygote is surrounded by a hard impermeable zygospore wall. During this time the chloroplast disintegrates, and lipid bodies accumulate. In the wild the zygospore can remain viable for several years. However, in the laboratory germination can be brought about after only a few days if nitrogen levels are restored in the presence of light. In most strains meiosis is followed by the release of four haploid cells, although in some strains meiosis is followed by mitosis, leading to the release of eight haploid cells from the zygospore. On rare occasions a diploid is released from the zygospore and begin to divide mitotically. Vegetative diploids can be selected by auxotrophic complementation (Harris 2001).

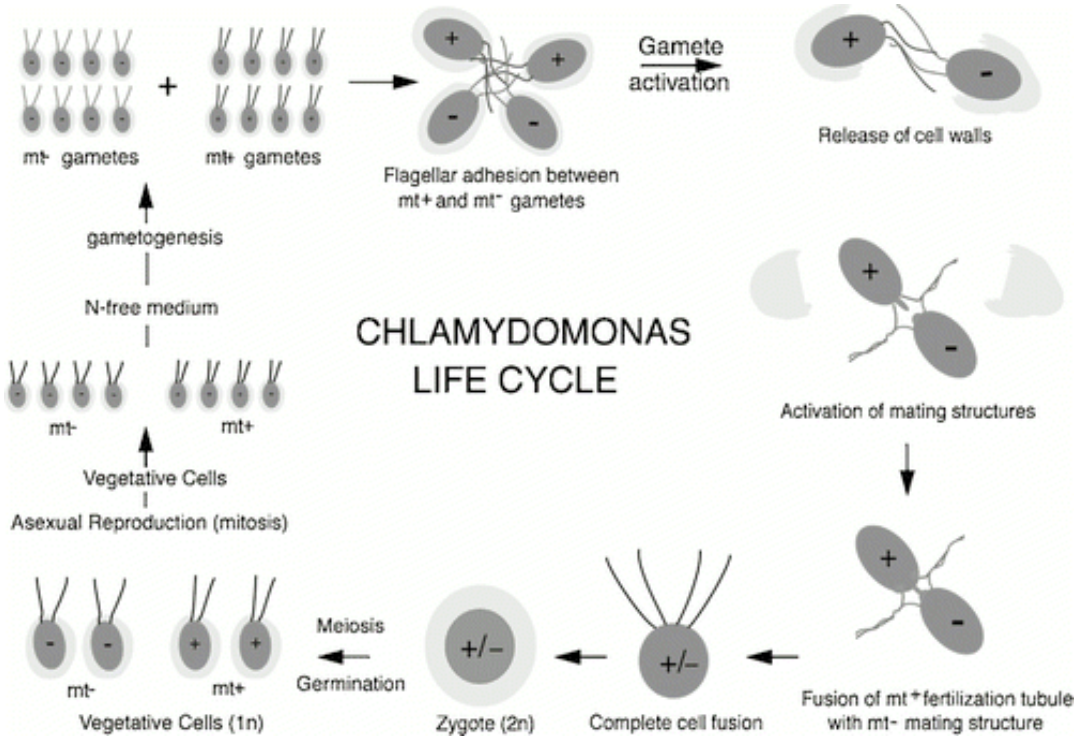


Figure 5 The life cycle of *Chlamydomonas*

The figure shows the various stages in the life cycle of *Chlamydomonas* described in the text above. Taken from Harris 2001.

Chlamydomonas has become a popular model organism for studying phenomena such as photosynthesis, plastid development, and flagellar biosynthesis, among others. Features that make *Chlamydomonas* suitable for the laboratory is the ease with which it can be cultivated, and its short generation time. Photoautotrophic cells can be grown on a simple medium, and under optimal conditions they divide every 6-8h. The isolation of non-photosynthetic mutants is possible since the cells can utilize acetate as their sole carbon source. Furthermore, both diploids and haploids can be made to grow vegetatively. The segregation of the products of meiosis in asci simplifies genetic analysis. Finally, methods have been devised for transforming both the nuclear and chloroplast genomes.

2. MATERIALS AND METHODS

2.1 Strains and media.

All cloning steps were carried out in the recombination deficient *E. coli* strain TB1.
Bacterial cells were grown in liquid LB medium or solid agar LB containing 60μg/ml ampicillin.
For transformation of the *Chlamydomonas* chloroplast the non-photosynthetic strain CC-373 (ac-u-c-2-21), obtained from the *Chlamydomonas* Genetics Center at Duke University, North Carolina, was used. This strain contains a 2.5 kb deletion comprising the 3´end of the chloroplast-encoded *atpB* gene and parts of the IR (fig. 6) (Blowers *et al*. 1989).
The *Chlamydomonas* transformant MU7 (Salvador *et al*. 1993b) (fig. 6) was used as a control in the Northern blot assay. MU7 harbors an *rbcL*:GUS reporter gene construct inserted in its chloroplast genome between the *atpB* gene and the IR. The construct consists of bases –290 to +97 of the *rbcL* 5´ region fused to the *E. coli uidA* (GUS, β-glucurodinase) gene, terminated by the *psaB* 3´ end. This includes the putative *rbcL* promoter and RNA stabilizing sequence, but not the enhancer described in section 1.1.

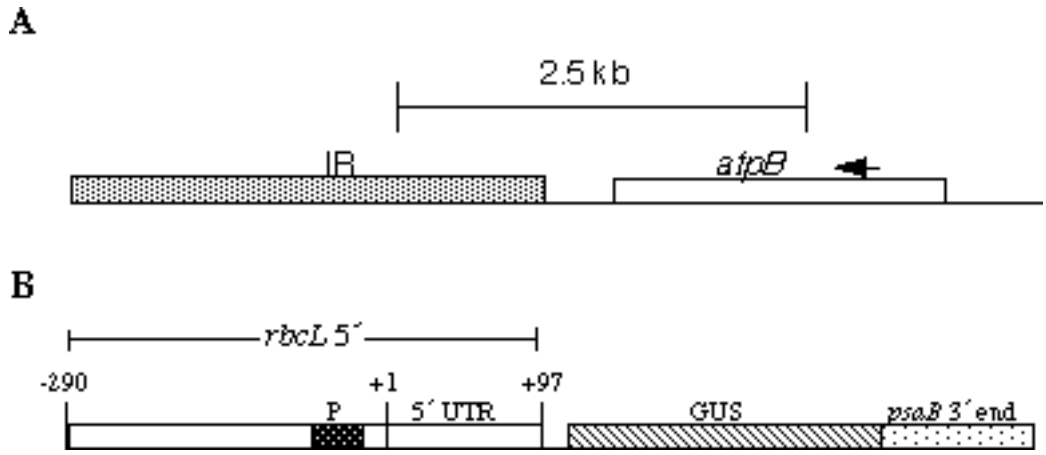


Figure 6 The *atpB* region of the chloroplast genome (A), and the MU7 construct (B).

(A) The *atpB* gene and the IR are represented by striped and open bars, respectively. The arrow designates the direction of transcription of the *atpB* gene. The 2.5kb deletion in mutant CC-373 is indicated by the line above the figure.
B) The *rbcL* 5´region, spanning bases –290 to +97, is shown as an open bar. A dark bar marked P represents the promoter. The gap between the *rbcL* 5´ and GUS is part of the cloning vector pBlueScript SK+ polylinker. The figure is not drawn to scale.

Non-photosynthetic mutants were grown in high salt (HS) medium (Sueoka 1960) supplemented with 2.5 g/L potassium acetate (HSHA). Prior to transformation the cells were incubated on a shaker at room temperature (RT) and diluted several times until they were in the log phase. Transformants were kept in HS medium in Erlenmeyer flasks at RT. For DNA isolation the cells were grown to high density in 100ml or 200ml tubes in a water bath at ~30º C under continuous illumination. The cultures were mixed by bubbling with air supplemented with 2% CO_2. For RNA isolation the cells were grown under identical conditions, but in a 12h light/12h dark regime.

2.2 Cloning of the pPHΔ 45-156 plasmid

2.2.1 Isolation, digestion, and ligation of the starting plasmid SK+157

The starting plasmid for generation of the end transformation vector used in this study was the ~5kb SK+157 plasmid (fig. 7). pSK+157 consists of the *Chlamydomonas rbcL* 5` terminal region from position –70 to +157 (transcription start at +1) cloned into pBluescript SK+ (Stratagene) upstream of the *E. Coli uidA* (GUS, β-glucurodinase) gene terminated by the *Chlamydomonas psaB* 3´ sequence. *Xho*I/*Xba*I sites flank the *rbcL* 5`;GUS chimera.

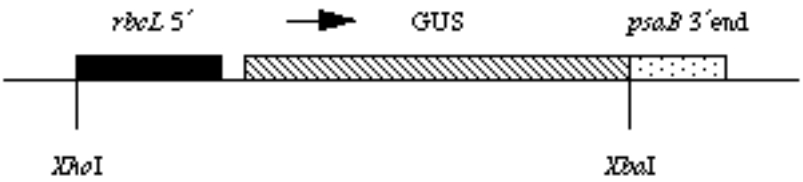
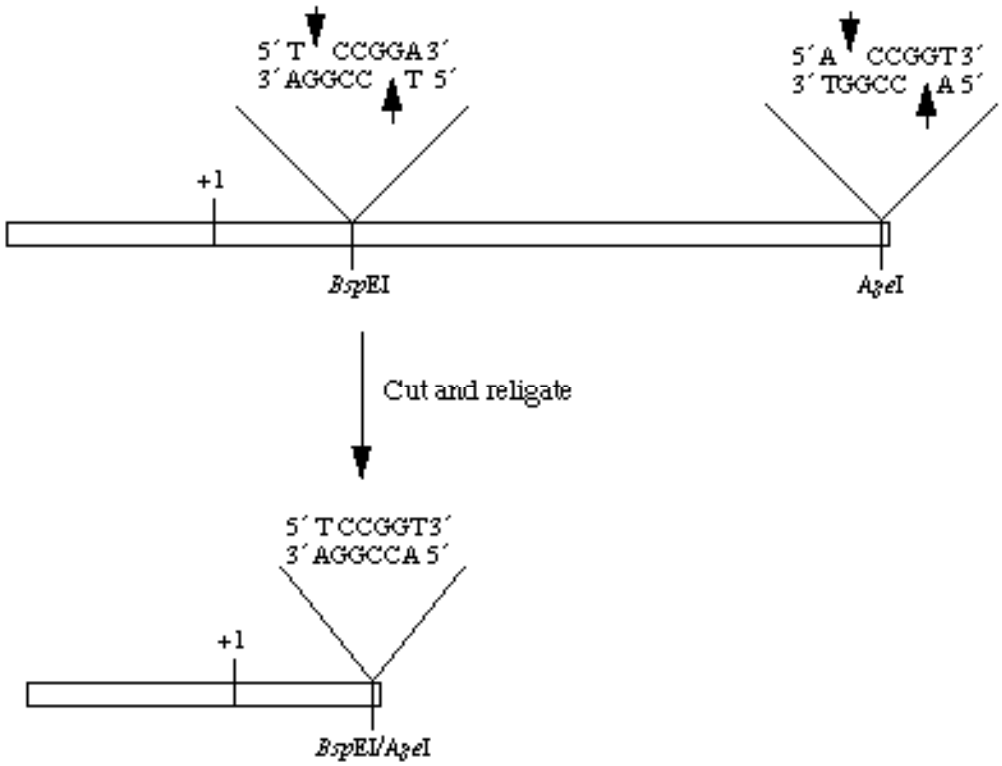


Figure 7 The *Xho*I/*Xba*I insert in SK+157

The arrow designates direction of transcription. Gap between GUS and *rbcL* 5´ UTR indicates that they are separated by part of the original pBluescript SK+ polylinker.

SK+157 was isolated from *E. coli* harboring the plasmid according to the maxiprep protocol (Sambrook and Russel 2001) and quantified by photospectrometry. To introduce a 110 bp deletion between position 45 and 156 in the *rbcL* 5´ UTR the plasmid was digested with restriction enzymes *Bsp*EI and *Age*I. *Age*I and *Bsp*EI recognize the six bp sequences 5´-ACCGGT-3´ and 5´-TCCGGA-3´, respectively, and both leave 4 nt 5´-CCGG-3´ 3´-overhangs. After digestion, the two fragments that resulted were separated on a 1% agarose gel, and the larger fragment was isolated from the gel. Briefly, a well is made in the gel downstream of the band to be isolated, and the downstream wall and the floor of the well is made DNA impermeable by a piece of dialysis tubing. The gel is then partially submerged in the running buffer, and the well is filled with buffer. Subsequently the DNA is electophoretically transferred to the buffer in the well, and removed with a pipette. Finally, the DNA is purified by phenol/chloroform extraction and ethanol precipitation.
The DNA was quantified by the dot spot method (Sambrook and Russel 2001). 80ng of the plasmid was religated with T4 DNA ligase at RT for two hours in the presence of 6% PEG. This was possible because *Age*I and *Bsp*EI leave overhangs that are complementary to each other. In effect this re-creates plasmid SK+157 with a deletion of all material between position +47 and the GUS sequence and point mutations from A to T at position +46 and from C to T at position +47 in what is now the *rbcL* 5´ UTR (fig. 8). In other words, what was bases +157 and +156 has now become bases +47 and +46.

A



B

AAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATT**TCCG**
GACAGATTATTTTAGGATCGTCAAAAGAAGTTACATTTATTTA
TATAAATGGTTCCACAAACAGAAACTAAAGCAGGTGCTGGATT
CAAAGCCGGTGTAAAAGACT**ACCGGT**T

C

AAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTTCCG
GTT

Figure 8 The *rbcL* 5´ UTR sequence before and after ligation

A) shows a schematic representation of the changes introduced in the *rbcL* 5´ region. The top panel shows the *rbcL* 5´ region before digestion. Restriction sites *Bsp*EI and *Age*I are shown along with their recognition sequences. +1 designates the transcription start site. The lower panel shows *rbcL* 5´ region after religation. The sequence that results from religating the *Bsp*EI and *Age*I 4 nt overhangs is shown. **B**) shows the first 157 bases in the *Chlamydomonas rbcL* 5´ UTR sequence before digestion and ligation. Restriction sites (*Bsp*EI and *Age*I) are in bold type. Bases deleted after digestion are underlined. **C**) shows the new *Chlamydomonas rbcL* 5´ UTR sequence after religation. Mutated bases are underlined.

2.2.2 Transformation and control of plasmid

After ligation 3 µl of the ligation mix (24 ng DNA) was used for transformation of $CaCl_2$ competent *E. coli* cells by the heat shock method (Sambrook and Russel 2001). Transformed cells were plated on solid ampicillin containing LB agar for selection (pBluescript SK+ contains a ampicillin resistance gene), and grown over night (ON) at 37° C. Colonies were picked and grown in liquid LB medium containing ampicillin ON at 37° C. Plasmid was isolated from the ON cultures according to the miniprep protocol (Sambrook and Russel 2001). To verify that the cloning had proceeded as planned, two separate test-cuts were made. First the plasmid was cut with *Swa*I. The unmodified SK+157 was also cut as a control. A single *Swa*I site is situated immediately downstream of the promoter in the untranscribed region of the *rbcL* 5´flanking region, so both the plasmid and the control would be expected to be cut once by this enzyme, producing ~5kb linear fragments. The control should be 110 bp larger than the plasmid. In a separate test-cut, both the plasmid and the control were cut with *Bsp*EI. *Bsp*EI would be expected to cut the control once, but not to cut the plasmid since the cutting and religation from the cloning destroys the original *Bsp*EI site. Plasmids from two different colonies were tested together with the control, and the products of all the test-cuts were run on the same 1% agarose gel (fig. 9) against a 1kb Plus ladder (Gibco BRL). Bands were visualized by EtBr staining.

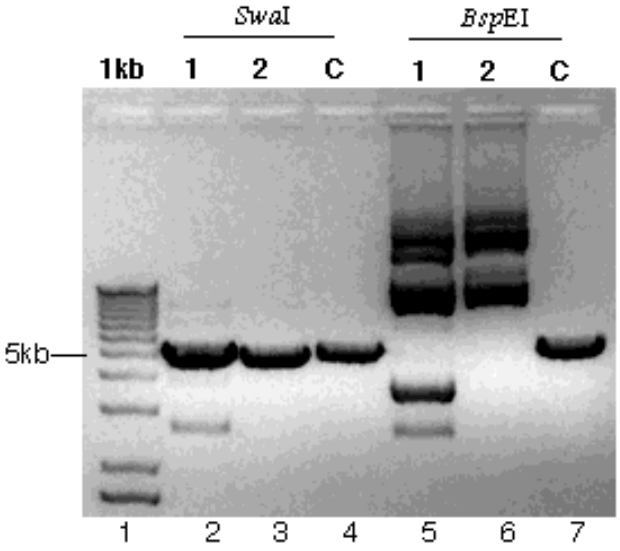


Figure 9 Test digestion of modified SK+157

The picture shows a photo of a gel with DNA from test-cuts described in the text above. Above the gel colony numbers and enzymes used are indicated. Beneath the gel picture the lane numbering is given. Lane one shows the 1kb Plus ladder. Lanes 2, 3, and 4 show test-cuts of colony 1, 2, and control, respectively. Likewise, lane 5, 6, and 7 show test-cuts of colony 1, 2, and control, respectively. Lanes 2 through 4 show test-cuts with *Swa*I, and lanes 5 through 7 show test-cuts with *Bsp*EI. Lanes 2 and 3 both show that the plasmids from the two colonies were cut once by *Swa*I, producing ~5kb bands. In lane 4 the control has been cut once

by *Swa*I. As expected, this fragment is slightly larger than the ones in lane 2 and 3. Lane 5 and 6 both show that the plasmids from the two colonies were not cut by *Bsp*EI (the plasmid DNA in lane 6 seems to be mostly un-super coiled). The control in lane 7 has been cut once by *Bsp*EI, producing a band identical to the one in lane 4. In lane 2 and 5 two unexplained bands at slightly less than 3kb can be seen.

From the gel picture above the plasmid from colony two was found to be correct, and this plasmid was used for subsequent cloning steps.

2.2.3 Cloning of the reporter gene cassette into the *Chalmydomonas* transformation vector

The plasmid from colony two was isolated by the maxiprep method as before and quantified by spectrophotometry. *Xho*I/*Xba*I was used to liberate the entire *rbcL*:GUS cassette, which was gel isolated as described earlier. The dot spot method was used for quantification.
The *Chlamydomonas* chloroplast transformation vector p +10 that has been developed in our laboratory consists of a chimeric *rbcL* 5´ region:GUS:*psaB* 3´ end gene flanked by part of the *Chlamydomonas* IR on the 5´ side, and the entire *Chlamydomonas atpB* gene on the 3´side inserted into the pUC8 vector (fig. 10). The p +10 plasmid *rbcL* 5´ region spans bases –70 to +157, and has changes introduced in the *rbcL* 5´ UTR between position +10 and +18 that introduces a *Sph*I site at position +18.

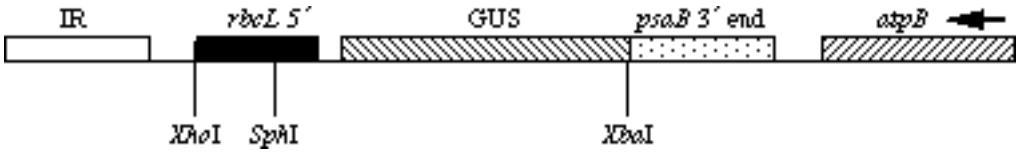


Figure 10 Plasmid p +10

The figure shows the insert in pUC8. *Xho*I/*Xba*I restriction sites used to liberate the *rbcL* 5´ region:GUS cassette and the *Sph*I site used in control of plasmid pPHΔ 45-156 are shown. The arrow above the *atpB* gene indicates direction of transcription.

To release the ~2kb *rbcL* 5´ region:GUS cassette, p +10 was treated with *Xho*I/*Xba*I, and the large (~8.5kb) fragment was gel isolated and quantified as before. The *rbcL*:GUS cassette isolated from colony two was ligated to the p +10 ~8.5kb fragment with T4 DNA ligase as described before, creating the transformation vector pPHΔ 45-156 (fig. 11). For ligation an insert:vector ratio of 1.3 was used. 3 µl of the ligation mix was used to transform *E. coli* cells as described in section 2.2.2. Transformed cells were plated on solid LB agar, and colonies were picked and grown in liquid LB medium ON as before. Plasmids were isolated from the ON cultures according to the miniprep protocol.

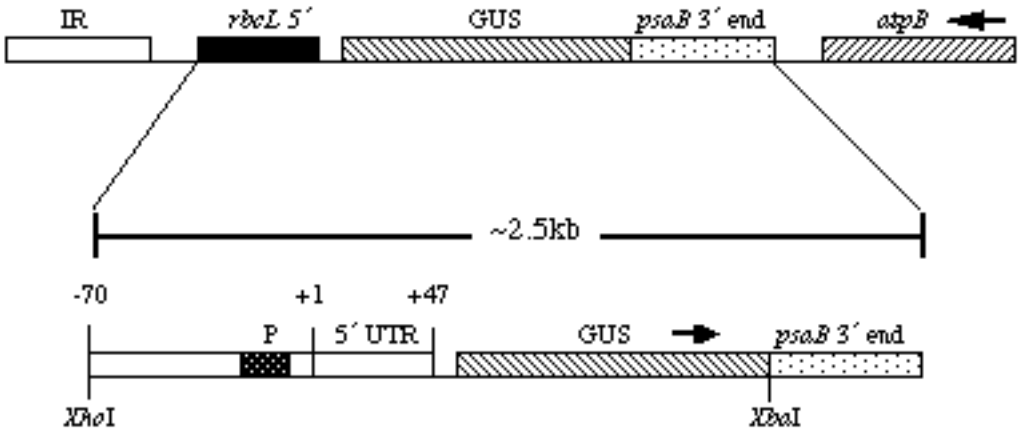


Figure 11 Plasmid pPHΔ 45-156

The figure is a schematic representation of the sequence inserted into pUC8 to yield plasmid pPHΔ 45-156. The lower section gives a more detailed view of the *rbcL* 5´:GUS:*psaB* 3´ cassette. The *rbcL* 5´ region spans bases between positions –70 and +47, with the transcription start point taken as position +1. The dark bar around position +10 marked P represents the promoter. The gap between the *rbcL* 5´ region and GUS indicates that these are separated by a sequence from the original pBluescript SK+ vector. Restriction sites used in sub-cloning and control of plasmid are shown. Arrows indicate direction of transcription. The figure is not drawn to scale.

2.2.4 Verification of final plasmid

The miniprep-isolated plasmids from section 2.2.3 were digested in two separate reactions, one with *Sph*I, and one with *Xho*I/*Xba*I. After digestion the products were separated on a 1% gel (fig. 12).

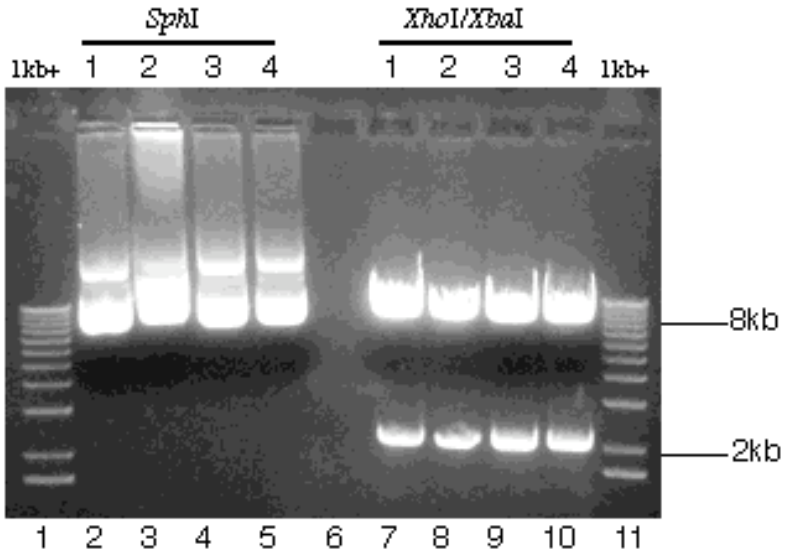


Figure 12 Test digestion of pPHDΔ 45-156

Lanes 1 and 11 show the 1kb plus ladder. Lanes 2 through 5 show plasmids from colony 1 through 4 digested by *Sph*I (indicated above gel; lane numbering below gel). Plasmids 1 through 4 digested by *Xho*I/*Xba*I are shown in lanes 7 through 10. As expected none of the plasmids were cut by *Sph*I, while all were cut once each by *Xho*I and *Xba*I, respectively,
producing the expected bands of ~2kb and ~8.5kb.

Plasmids from four colonies were tested. The *rbcL* 5´ region:GUS insert in p +10 contains an *Sph*I site, while the *rbcL*;GUS cassette in pPHΔ 45-156 does not. So, if the original cassette in p +10 has been replaced, one would expect *Sph*I not to cut the plasmid. Digestion of pPHΔ 45-156 with *Xho*I/*Xba*I should produce two fragments, the ~2 kb *rbcL*:GUS cassette and the ~8.5kb p +10 sequence.
All four colonies were found to harbor the correct plasmid, and one was chosen at random for further use. After isolation of plasmid from an ON culture by the maxiprep method, the plasmid was sequenced to verify that the correct modifications had been introduced (fig 13).

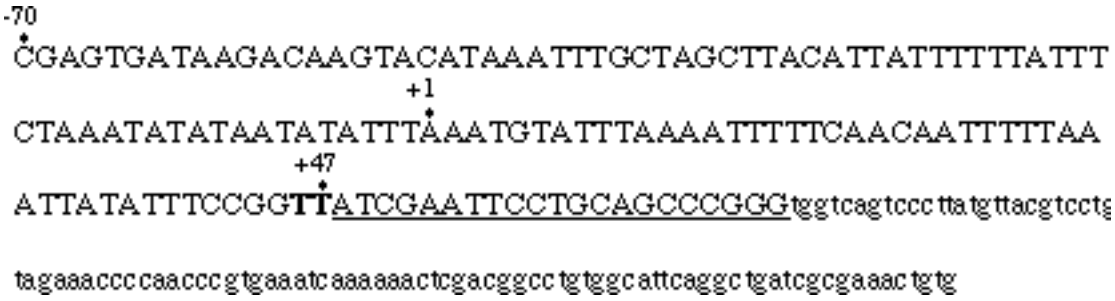


Figure 13 Readout from sequencing

The figure shows the sequencing of pPHΔ 45-156. Positions –70, +1, and +47 are indicated. Changes introduced into the rbcL *5´ region are in bold type. SK+ vector sequence is underlined, and GUS sequence is in lower cast.*

2.3 Chloroplast transformation.

Transformation vector pPHDΔ 45-156 was introduced into the chloroplast of *Chlamydomonas* strain CC-373 by particle bombardment essentially as described by Boynton *et al*. (1988) and Blowers *et al*. (1989). Cells were bombarded with DNA precipitated onto 0.6 μm gold microparticles. The microparticles were accelerated to high velocity by a burst of helium gas at a pressure of 1350 psi with the PSD-1000/He particle delivery system from BioRad. The shooting distance was 15 cm. Prior to transformation, CC-373 cells in the log phase of growth were transferred to HSHA agar plates at high density. Plates were kept in the dark at room temperature for four hours prior to shooting. Once inside the chloroplast, part of the vector is integrated into the genome by homologous recombination (fig. 14). After shooting, the cells were kept in the dark for one day to allow this to happen. Next, the cells were transferred to HS agar plates and placed under bright light.

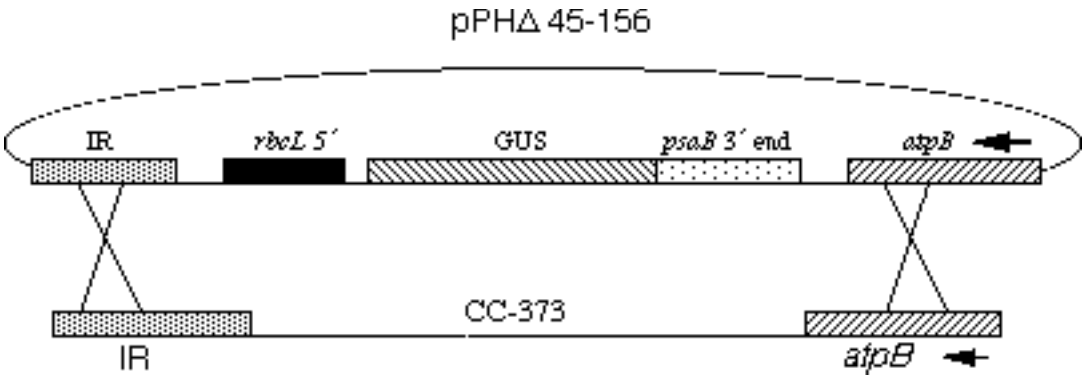


Figure 14 Recombination between transformation vector pPHΔ45-156 and the chloroplast chromosome of *Chlamydomonas* mutant strain CC-373.

The figure shows the double recombination event between the IR and *atpB* regions of the transformation vector and deleted IR and *atpB* sequences in strain CC-373. This recombination event restores the IR and the atpB gene of CC-373 to the wild-type sequence with the concurrent introduction of the reporter-gene cassette, thus restoring the photosynthetic capabilities of the cells. Drawing is not to scale.

Recombination between the vector and the chloroplast genome restores the deletion in the *atpB* gene of the mutant, re-establishing the photosynthetic capacity of the cells. This forms the basis for selection of transformants, since only photoautotrophic cells are able to survive on HS medium. Two weeks after

shooting transformed cells can be seen as green colonies among the dead, white, untransformed cells on the HS plates.
Unfortunately, recombination between vector and chloroplast genome can sometimes restore the deletions in the IR and *atpB* gene of the mutant without integrating the reporter gene construct. Any given transformant may therefore contain chloroplast genomes both with and without the reporter gene construct. This makes it necessary to determine the GUS content in the cell lines from each colony.

2.4 Slot blot analysis.

An initial screening of colonies for GUS content was carried out by the slot blot method as described by Blowers *et al.* (1990). Total DNA was isolated as described by Dellaporta *et al.* (1983), quantified by the dot spot method as before, and 700 ng was blotted onto a nylon membrane (ZetaProbe; BioRad) using a Hoefer slot blot apparatus. The membrane was probed with a radiolabeled GUS probe generated by the random primer labeling method (Feinberg and Vogelstein 1983) with α-^{32}P-dCTP as the radiolabeled nucleotide, and the *Bam*HI-*Sac*I GUS fragment from plasmid pBI221 (Clontech, Palo Alto, California) as the double stranded template. Hybridization and washing was carried out as described by Church and Gilbert (1984). The hybridization time was 24h. Membranes were exposed to X-ray film (Biomax MS; Kodak) at -80°C over night with an intensifying screen.
The slot blot procedure only gives a qualitative description of the GUS content of a transformant since the strength of the signal depends on factors such as exposure time, specific activity of the probe, etc. However, by comparing the signal strengths the procedure can be used to determine the relative GUS content in each sample.

2.5 Southern analysis.

A restriction fragment length polymorphism results from the differential integration of the GUS reporter construct into the chloroplast genome. This polymorphism can be exploited to give a quantitative description of the degree of homoplasmicity, with regard to the presence of the GUS sequence, for a transformant by a Southern blot analysis (see 3.2 section for details).
The Southern blot procedure was first described by E.M. Southern (1975). Since then many variants have been developed, but the basic principle remains the same. DNA is isolated, digested, and separated according to size on a gel, and the DNA is transferred to a membrane, usually by capillary pull. The membrane is then probed by a labeled oglionucleotide complementary to the DNA sequence one wishes to detect.
In this study total DNA was isolated as described in section 2.4, and 2μg was digested by *Kpn*I and *Hin*dIII. The resulting restriction fragment mixture was

separated on a 1% agarose gel with the Gibco BRL 1kb Plus ladder as a size marker. A picture was taken of the gel for later size-determination of hybridization bands.
The DNA was then transferred to a ZetaProbe membrane by the alkaline blotting procedure as described in the BioRad ZetaProbe manual.
The probing, washing, and exposure procedures were as described for the slot blot.
The hybridization probe was the random primer labeled *atpB* sequence generated with the *Hpa*I/*Eco*RV fragment from plasmid pCrc*atpB* (Blowers *et al.* 1990) as the template.

2.6 Northern analysis.

For detection of reporter gene transcripts the Northern blot procedure was employed.
Northern blots are similar to Southern blots, except the nucleic acid to be transferred to the membrane is RNA rather than DNA. In addition, RNA´s tendency to form secondary structures requires denaturing conditions to be applied.
Here, total cellular RNA was isolated as described by Merchant and Bogorad (1986), and the concentration was calculated from the samples` UV-light (260nm) absorption according to the formula: total mass of RNA [µg]=360xAbs. Four µg RNA were separated on a 1.3% agarose/formaldehyde gel as described by Blowers *et al.* (1990). Transfer of RNA to a nylon membrane (ZetaProbe; BioRad) was done according to the manufacturer`s manual.
The membrane was probed with a GUS probe, and exposed to X-ray film, as described for the slot blot.

3. RESULTS

3.1 Relative GUS content of transformants.

Transformation of strain CC-373 with the pPHDΔ 45-156 vector gave six colonies, designated colonies PHΔ 45-156 one through six. All of these were

screened for the presence of the GUS sequence. From the autoradiography (fig. 15) colony five can clearly be seen to have the highest GUS content, and this was chosen for further analysis.

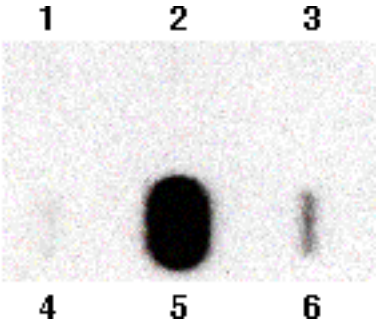


Figure 15 Autoradiogram from slot blot

DNA from colony 1 through 6 was blotted onto a nylon membrane and probed with a radiolabeled GUS probe. Samples from colony 1,2,3, and 4 gave no signal. For DNA isolated from cells of colony 6 a faint signal can be seen. DNA isolated from transformants in colony 5 gave the strongest signal, and was selected for further use.

3.2 Quantitative determination of GUS content

As mentioned earlier, transformation, and subsequent selection for the presence of the *atpB* gene under bright light, can lead to the formation of two types of chloroplast genomes. The introduction of the wild type *atpB* gene to mutant CC-373 both alone, and together with the GUS reporter gene construct, restores the photosynthetic properties of the cell. There is therefore no selection for the presence of the GUS sequence in the transformation procedure used here. As a result the 50-80 copies of the chloroplast genome in one cell may consist of a mixture of DNA molecules both with and without the *rbcL* reporter-gene chimera. In addition, the ratio between the two types of genomes in the different cells in a culture generated from one transformant can vary.
When these two types of chloroplast genomes are digested by *Kpn*I and *Hin*dIII *atpB*- containing fragments of two different sizes are produced (fig. 16).
*Kpn*I makes a cut in the IR sequence just upstream of the *rbcL* promoter, while *Hin*dIII cuts immediately upstream of the *atpB* promoter. If the *rbcL*;GUS chimera is included this creates a ~5.4 kb fragment. Without this insert a ~3 kb fragment results. The ratio of the intensities of the 5.4 and 3 kb bands on a Southern blot gives the ratio between the two types of chloroplast genomes in a culture.

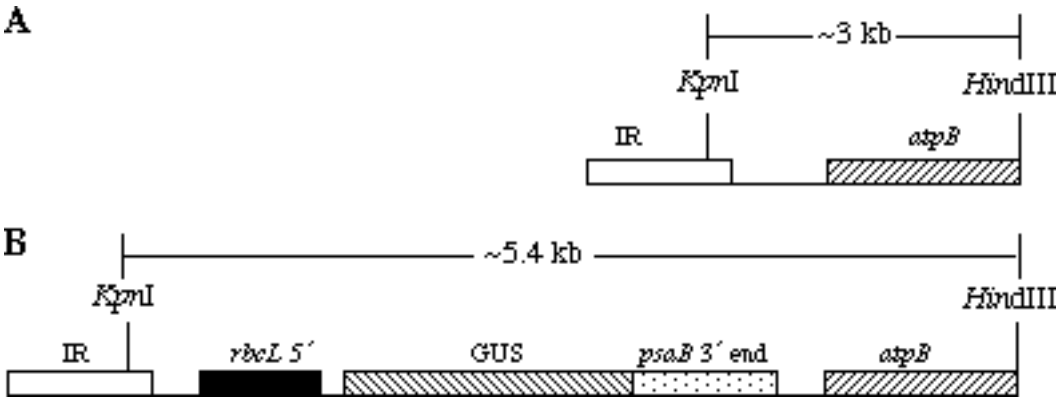


Figure 15 atpB containing restriction fragments

The *atpB*-containing *Kpn*I/*Hin*dIII fragments produced when cutting chloroplast DNA without (**A)** and with (**B**) the GUS containing reporter gene sequence. When the reporter gene is present a ~5.4 kb fragment is created, and when this is not present a ~3 kb fragment is produced. Not drawn to scale.

Both PHΔ 45-156 transformant five, selected in the slot blot, and the MU7 control were subjected to Southern analysis. From the autoradiograms (fig. 17) the MU7 can be seen to be homoplasmic for the reporter gene construct, i.e. only chloroplast chromosomes with the *rbcL*:GUS chimera integrated are present. The pPHDΔ 45-156 seem to have the reporter gene construct integrated in 60 - 70% of its chloroplast genomes, while the remaining 30 - 40% do not harbor the insert. Although a homoplasmic PHΔ 45-156 transformant would be desirable, this transformant can still be used as long as one takes the result from the Southern into account when interpreting the Northern blot (see section 3.3 for details).

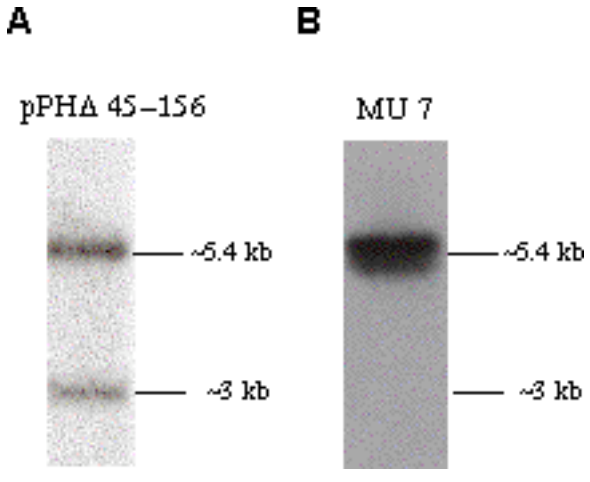


Figure 17 Southern blots

The figure shows autoradiograms from Southern analysis of PHΔ 45-156 (**A**) and MU7 (**B**). Total cellular DNA was digested by *Kpn*I and *Hin*dIII, separated on a 1% agarose gel, and transferred to a nylon membrane (ZetaProbe; BioRad). The membrane was probed with an *atpB* sequence containing radiolabeled probe, and bands were visualized on an X-ray film (Biomax MS; Kodak) by over-night autoradiography at -80°C with an

intensifying screen. The MU7 can be seen to only produce a ~5.4 kb band. The ~5.4 kb band accounts for 60 - 70% of the total staining of the film from the PHΔ 45-156, while the ~3 kb band accounts for the remaining 30 – 40%.

3.3 Reporter gene transcript accumulation.

To compare the steady-state accumulation of GUS transcripts between the MU7 control and the PHΔ 45-156 construct, a Northern blot analysis was performed. This was done both for light-grown (RNA isolation 1h into the light period of the 12h light/12h dark regime) and dark-grown (RNA isolation 11h into dark period) cells.
In a Northern blot the degree of staining of the x-ray film from one sample is directly proportional to the amount of target RNA in that sample. To ensure that the signal from each sample is directly comparable it is important that the amount of RNA loaded on the gel is the same for all samples. This can be verified by inspecting the intensities of the bands in the EtBr stained gel (the bands seen on the gel are primarily rRNA, which make up the bulk of the RNA pool in the cell. The amount of mRNAs, like the GUS transcript investigated here, is too small to be seen as discrete bands on the gel). Should the amount of RNA loaded from each sample differ, this must be taken into account when interpreting the final result. In addition to this, the signal from non-homplasmic transformants must be normalized relative to the signal from homolasmic transformants.
From the gel picture below (fig. 18), the amount of RNA loaded on the gel for both the MU7 and the PHΔ 45-156 can be seen to vary in an acceptable range.

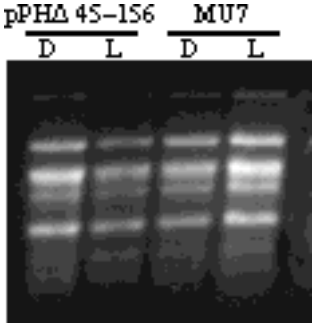


Figure 18 Northern gel

Photography of EtBr stained Northern gel. Dark-isolated (D) and light-isolated (L) RNA samples of the MU7 and PHΔ 45-156 were run on an agarose/formaldehyde gel in the order indicated above the figure. The amount of RNA in the four lanes can be seen to vary in an acceptable range.

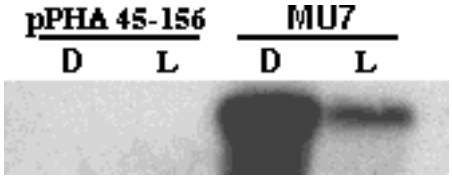


Figure 19 Northern autoradiogram.

The figure shows the autoradiogram from the Northern analysis. Dark (D) and light (L)-isolated RNA samples (4μg) from transformants MU7 and PHΔ 45-156 were separated on a 1.3% agarose/formaldehyde gel, and transferred to a nylon membrane (ZetaProbe; BioRad). The membrane was probed with a P^{32}-labled GUS probe. Hybridization bands were visualized by overnight exposure to

an X-ray film (Biomax MS; Kodak) at -80°C with an intensifying screen. A strong signal can be seen from the MU7 dark-sample, whereas the signal form the MU7 light sample is only ~30% of the dark-sample. No signal was detected from the two PHΔ 45-156 samples.

The autoradiogram from the Northern blot (fig. 19) reveals a strong signal from the dark-isolated MU7 sample, indicating that the GUS transcript is accumulated to a relatively high level in this construct. From the light-isolated MU7 sample, a signal approximately 30% of that of the dark-sample can be seen. No signal was detected from the light- nor the dark-isolated PHΔ 45-156 sample.
Although the PHΔ 45-156 transformant was only ~70% homoplasmic, one would expect to be able to detect a signal from this construct, had it accumulated the GUS transcript at levels comparable to the MU7. Consequently, it is concluded that the PHΔ 45-156 transformant does not accumulate GUS.

4.DISCUSSION

From the data presented here alone, it is not possible to decide whether the failure of transformant PHΔ 45-156 to accumulate the GUS transcript stems from a problem with transcription or RNA stability. However, taken together with the results presented by other workers, it seems likely that this failure is a result of reduced RNA stability, and not a reduced rate of transcription. First of all, the same putative promoter elements are present in PHΔ 45-156 and MU7 (Fig. 20). Both retain the –10 TATAATAT element described in section 1.1. This element has been shown to be sufficient for basal levels of transcription to occur. It has also been shown that no basal promoter elements reside in the 5´ UTR of the *Chlamydomonas rbcL* gene (Klein *et al.* 1994, Salvador *et al.* 2004). In addition, both lack the enhancer-like element located in the *rbcL* coding region, between position +108 to +143. This element is necessary for maximum transcription (Anthonisen *et al.* 2002). From this alone, one would expect the two transformants to transcribe the reporter gene at comparable rates.

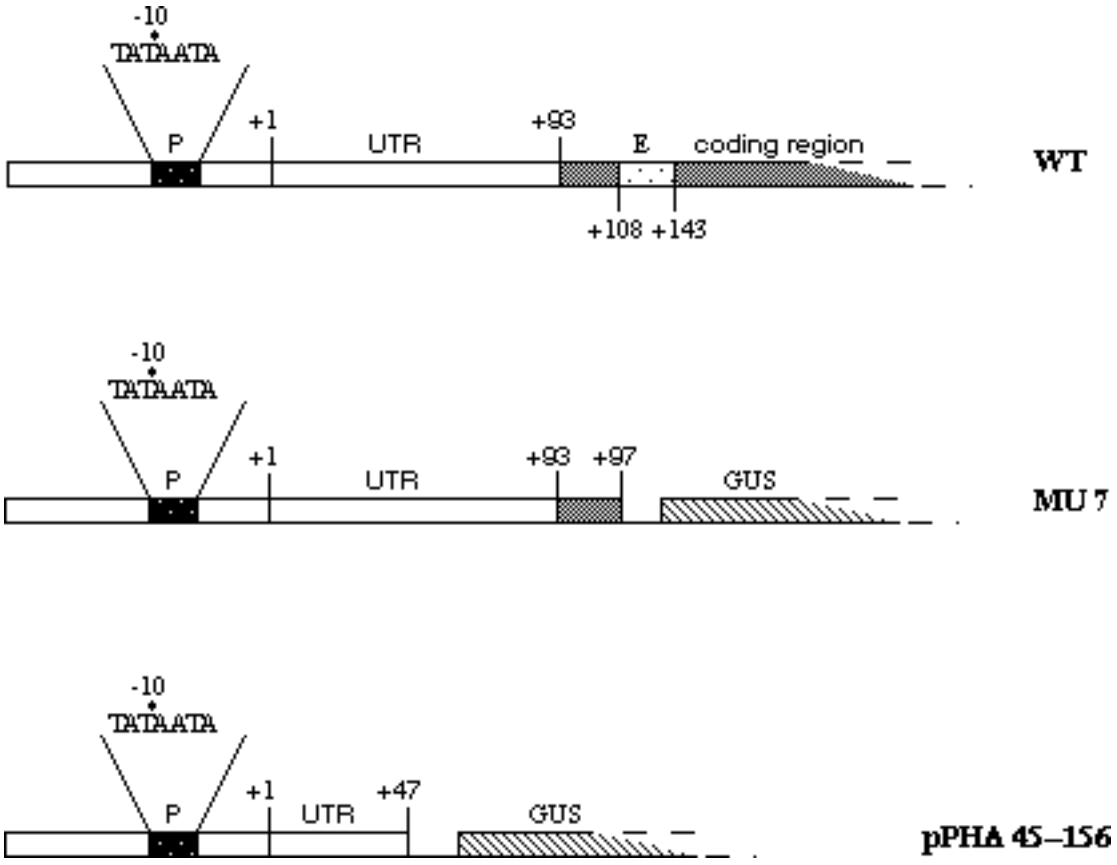


Figure 20 Transcription-promoting elements in wild type *rbcL* and constructs MU7 and PHΔ 45-156

The figure shows the putative transcription promoting elements present in the wild-type (WT) *rbcL* gene and reporter gene constructs MU7 and pPHΔ 45-156. The promoter, at position –10 relative to the transcription start site, is marked P, and its sequence is shown above the figures. The enhancer-like element situated between position +108 to +143 is represented by a dotted box, and is marked E. From the figure one can see that the promoter sequence is present in all three segments, while both MU7 and pPHΔ 45-156 lack the enhancer-element.

The changes induced in *rbcL* 5´UTR during the assembly of the pPHΔ 45-156 construct affect the afore mentioned RNA stabilizing structure (section 1.2). This structure comprises the first 46 nt of the UTR, of which the sequence of the last 9 nt is vital for the stability. The first 41 nt partake in the formation of a stem-loop (Fig. 21 **A**). Changing the sequence of the stem-loop does not alter the transcript stability, as long as the secondary structure is maintained, and the identity of the last 9 nt remains unchanged (Anthonisen *et al.* 2001, Uwe Klein, personal communication).
In construct pPHΔ 45-156 the sequence of the first 46 nt of the 5´UTR is the same as in the wild-type *rbcL,* except from a point mutation at position +46 from A to T. This, in effect, changes the last A in the 9 nt stabilizing sequence to a U. An mfold simulation (Zuker *et al.* 1999) of the first 46 nt of the RNA sequence

of the pPHΔ 45-156 5UTR predicts that the stem-loop structure is unchanged in the PHΔ 45-156 transcript, relative to that of the wild-type. (Fig. 21 **B**).

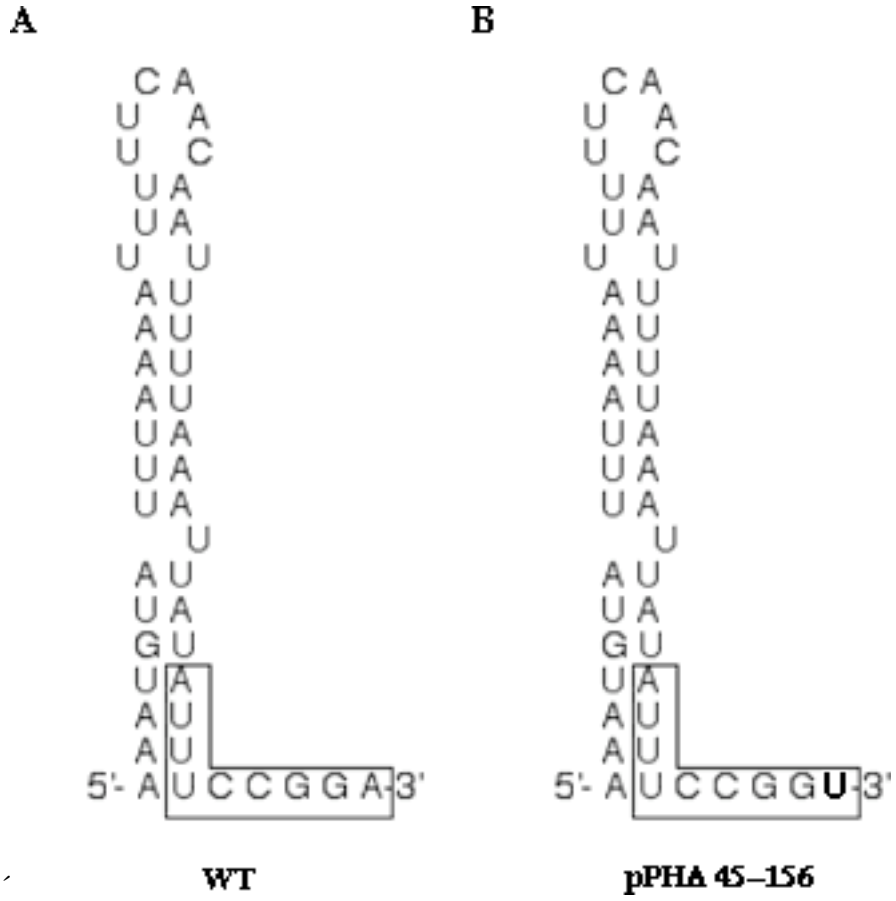


Figure 21 Secondary structure of the *rbcL* 5´ UTR

The figure shows the predicted structure of the wild type (**A**) and PHΔ 45-156 (**B**) transcript 5´UTRs. The 9 nt stabilizing sequence in the wild type is indicated by an open box. The corresponding sequence in PHΔ 45-156 is also boxed, and the A to U change is shown in bold type. Adapted from Salvador *et al*. (2004).

The change from C to T at position +47 is outside of the 9 nt stabilizing element. It is therefore not likely that this change influences the stability of the transcript. To ensure that the 110 bp deletion, which moves the GUS sequence closer to the stabilizing stem-loop, does not change the secondary structure of the first 46 nt of the transcript a mfold simulation of the first 164 nt of the transcript was done (not shown). This includes the first 47 nt of the *rbcL* 5´ UTR, the SK+ vector sequence, and part of the GUS sequence. No changes in secondary structure were predicted.
Finally, it has been shown that the sequence downstream of position +55 in the *rbcL* 5´UTR is not important for transcript stability (Salvador *et al*. 2004).

Considering this, it seems unlikely that the reduction in reporter gene accumulation in transformant PHΔ 45-156 can be attributed to a reduced rate of transcription caused by the 110 bp deletion in the 5´UTR from position +45 to +156. Rather, it is more likely that the disruption of the 9 nt stabilizing element by the point mutation introduced at position +47 renders the reporter gene transcript susceptible to rapid degradation.

The results presented above point to a weakness in using the Northern blot procedure for mapping promoter elements in the type of reporter gene assay used here. Since the procedure relies on the reporter gene transcript to accumulate for detection of transcription, it is unable to distinguish between a lack of transcription and an unstable transcript. A method that is less sensitive to unstable transcripts is the *in vivo* labeling method described by Blowers *et al*. (1990). In brief, transformed cells are cultured for several generations on a low phosphate medium. The cells are then pulse–labeled with P^{32} inorganic phosphate, which is incorporated into all new transcripts. RNA is isolated, and GUS containing reporter transcripts are hybridized to GUS sequences immobilized on a nylon membrane. The RNA samples are extracted 10 and 20 min. after pulse-labeling, and transcription rates are calculated from the difference in signal strength between the 10 and 20 min. samples. Because the labeled transcripts have been synthesized since the addition of radiolabeled phosphate, these measurements reflect the dynamics of transcription rather than the steady state RNA levels, as is the case in the Northern blot procedure. If the half-life of reporter transcripts is in the range of a few minutes or more, this method will be able to detect the transcripts. However, if the transcripts are extremely unstable this method will also fail to produce a signal. Had this method been employed on the pPHΔ 45-156 transformant, it might have been possible to unequivocally determine whether its failure to accumulate the reporter transcript stemmed from a problem with stability. Nevertheless, due to practical considerations, this was not done.

The dark/light pattern of transcript levels observed for the MU7 transformant is in agreement with earlier reports (Salvador *et al*. 1993a). This study showed that the inclusion of the *rbcL* 5´UTR in a reporter gene renders its transcripts unstable in the light relative to in the dark. While the half-life of the endogenous *rbcL* gene is 21 and 4 h in the dark and light, respectively, the half-life of the *rbcL* 5´UTR-containing reporter gene construct in the light is dramatically reduced (it has a 16 times higher rate of degradation in the light relative to in the dark) (Salvador et al. 1993b). This is in accordance with the results presented here, with the signal from the light-isolated MU7 sample being only about 30% of that from the dark-isolated MU7 sample. As previously mentioned (section 1.2), Singh *et al.* (2001) found that the sequence between position +27 and + 41 in the stem-loop formed by the first 41 nt of the 5´UTR is the target for light induced RNA degradation of *rbcL* 5´ containing reporter transcripts (fig. 22**A**). This paper also describes a stem loop in the coding region of the endogenous *rbcL* gene, situated around position +327, whose loop portion suppresses the destabilizing effect of the +1 to +41 stem loop. Furthermore, it was postulated

that this stabilizing effect was the result of an interaction between the loop portions of the two stem-loops, possibly modulated by protein factors (fig. 22**B**).

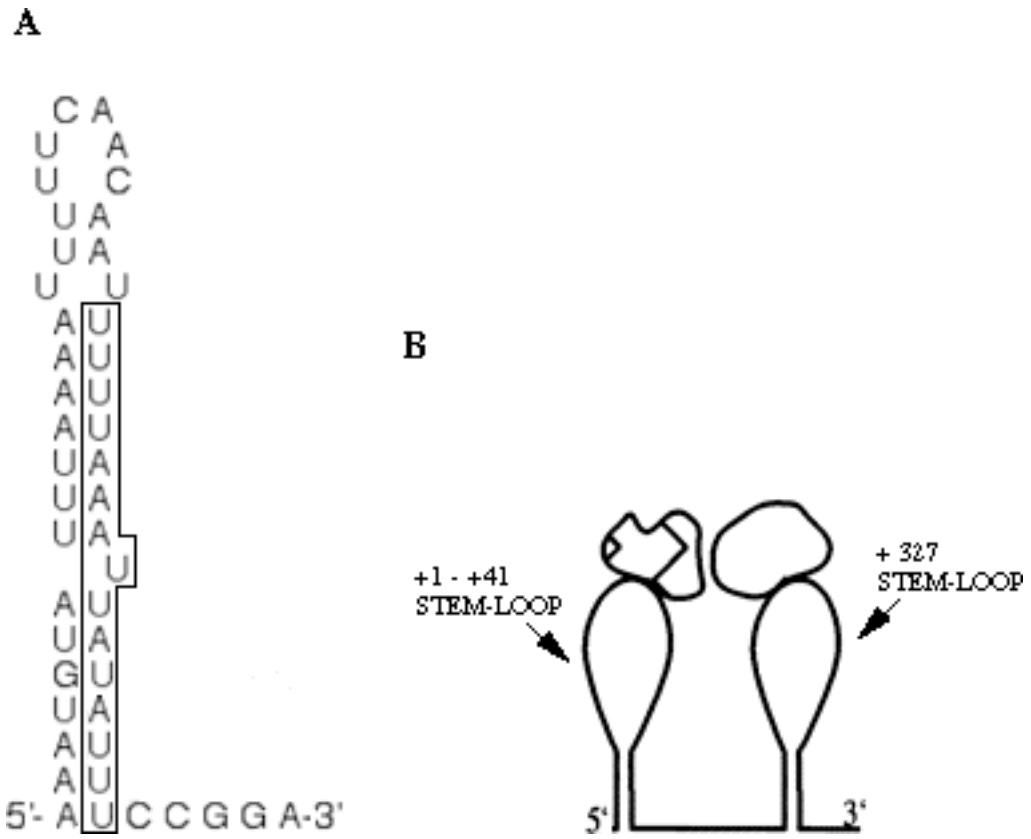


Figure 22 Light degradation target (A) and stem-loop interaction (B) in the *rbcL* gene.

A) shows the first 46 nt of the *rbcL* 5´UTR, including the stem-loop made up of the first 41 nt. The boxed sequence is the proposed target for the light-induced degradation of *rbcL* 5´UTR-containing constructs (see text above for details). **B**) shows the hypothetical interaction between stem-loops in the 5´UTR and coding region of the *rbcL* transcript described in the text above. This interaction is believed to have a stabilizing effect on the transcript. The protein factors proposed to mediate this interaction are shown. **A)** adapted from Salvador *et al*. (2003), and **B**) adapted from Singh *et al*. (2001).

The mechanism behind the stabilizing effect of the first 46 nt of the *rbcL* 5´UTR on transcripts is not known, although it has been suggested that the 9 nt sequence between position +38 and +46 together with the stem-loop structure in general is the binding target for *trans*-acting factors, e.g. proteins that protect the transcript from degradation. This would resemble the situation described in section 1.2 for the *psbD* transcript. The *nac*2 nuclear mutant lacks a protein that has been found to indirectly interact with the chloroplast encoded *psbD* mRNA 5´UTR, and thereby stabilizing the transcript (Boudreau *et al*. 2000). *nac*2 is associated with a large protein complex, and a 47 kDa protein that binds the *psbD* mRNA 5´UTR

in the presence of *nac*2 (Nickelsen *et al*. 1994) has been proposed to mediate the interaction between the *psbD* mRNA 5´UTR and this complex. Likewise, the nuclear encoded Mbb1 protein is able to restore chloroplast *psbB* mRNA levels in nuclear mutants that fail to accumulate this transcript (Vaistij *et al*. 2000). These findings point to what might be a general mode of regulation of chloroplast genes. It is possible that the gene investigated here, the *Chlamydomonas reinhardtii rbcL* gene, is subject to the same mode of regulation. However, further investigation will be required to confirm or refute this hypothesis.

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