5’ UTR sequence requirements for stability of *rbc*L transcripts in the chloroplast of *Chlamydomonas reinhardtii*

Rachel Gershuny

Cand. Scient. thesis in biotechnology
Department of Molecular Biosciences
University of Oslo, June 2005

Acknowledgments

This work was carried out during the period of September 2002 to January 2004 at the University of Oslo, department of Molecular Biosciences, under the supervision of Professor Uwe Klein.

I would like to thank Professor Klein for his patient guidance and assistance in both the laboratory work and the writing of this thesis.

I would also like to thank my fellow students, as well as Ph.D student Emnet Abesha and Postdoc Anne K. Kristoffersen, for their help, support and very pleasant company.

Finally, I would like to thank my family and friends for their constant encouragement and understanding throughout this period. A special thank you to my husband for his endless patience.

Oslo, June 2005

Rachel Gershuny

Table of Contents

Abstract

A reporter gene was constructed of the *Chlamydomonas reinhardtii rbc*L 5' region from position -70 to +47, relative to the transcription start point (+1), followed by the *E. coli uid*A (GUS) gene and the *Chlamydomonas psa*B 3' region downstream of it.

Two constructs were made in order to examine the importance of the sequence of a predicted stem-loop structure between positions +1 and +41 of the *C. reinhardtii rbc*L 5' region. The first construct tested consisted of a reversed nucleotide sequence between positions +5 and +37, while the second construct examined consisted of a complete change of sequence between positions +6 and +36 of the region, in which the nucleotides were changed so that each purine is replaced by another purine (A→G; G→A), and each pyrimidine by another pyrimidine (C→T; T→C).

The constructs were inserted into the chloroplast genome downstream of the *atp*B gene. Transcript accumulation of the reporter gene was determined by Northern blot. Both constructs did not exhibit a change in accumulation of GUS transcripts in comparison to the original reporter gene construct, proving that the altered nucleotides are not significant in stabilizing the *rbc*L transcript in the *C. reinhardtii* chloroplast.

1. INTRODUCTION

1.1 The Chloroplast

The chloroplast is a specialized organelle found in photosynthetic organisms. The name of the organelle arises from the prominent pigment it contains, chlorophyll. Light excites the pigment molecules and their structure allows energy transfer to proteins that lie in complex with them. This energy is used for the process of photosynthesis, in which carbon dioxide (CO_2) and water are converted into carbohydrates and other compounds.

The chloroplast is organized with two membranes surrounding an inner space called stroma, which contains stacked membranes called thylakoids (fig 1.1). The thylakoid membranes contain unique protein complexes – photosystems I and II (pigment containing), cytochromes b and f, and ATP synthase. These protein complexes cooperate to produce the energy needed in the process of carbon fixation.

Chloroplasts belong to a large organelle group called plastids, and are the most prominent of this group. All plastids are semi-autonomous organelles, they contain their own DNA and replicate by division.

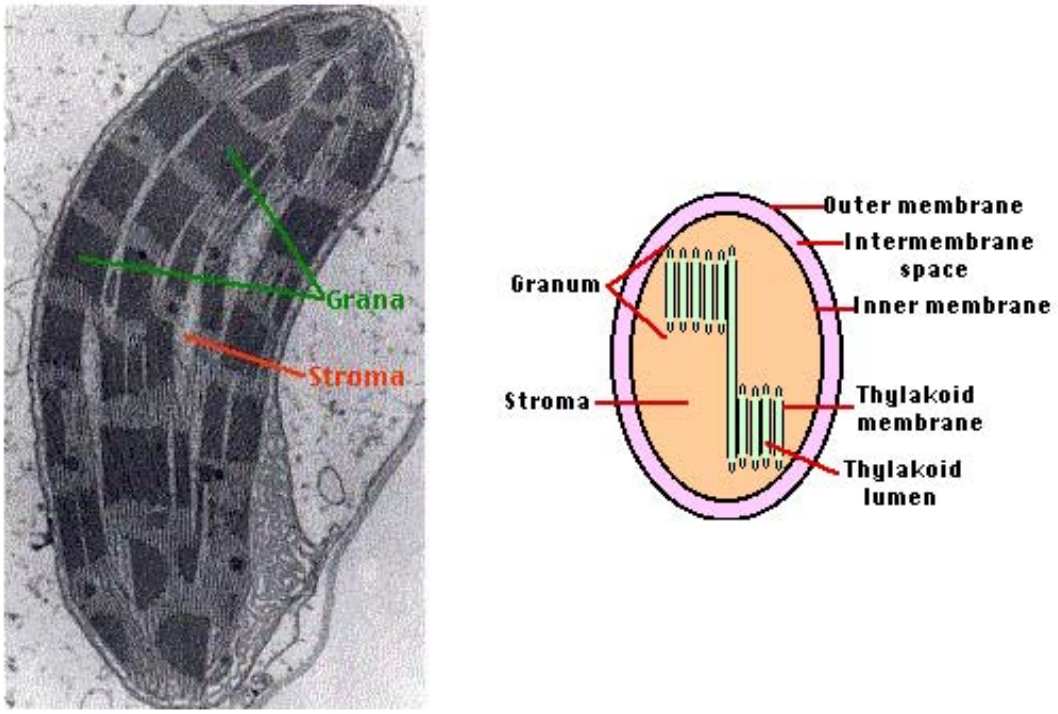


Fig 1.1 The chloroplast

A schematic look and an electron micrograph showing chloroplast organization. (http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Chloroplasts.html)

Chloroplasts are semi-autonomous organelles. This means that they contain genes necessary for the processes taking place in the organelle, as well as a system for the expression of these genes, but are not independent of nuclear gene products for their biogenesis and their function. Perhaps the most obvious example for this interdependency is the two RNA polymerases active in chloroplast transcription, one of them a chloroplast-encoded RNA polymerase (PEP) and the other a nuclear-encoded RNA polymerase (NEP).

A widely accepted explanation for this interdependency is the endosymbiot theory, which claims that chloroplasts originated from an ancestor of *cyanobacteria*. The theory suggests chloroplasts arose through a process in which a photosynthetic organism was engulfed by a non-photosynthetic organism, which then gained the photosynthetic ability. This theory relies on genetic similarities between chloroplasts and *cyanobacteria*. Plant chloroplast genomes are substantially smaller than cyanobacterial genomes. This is assumed to be the result of gene loss or gene transfer from the chloroplast to other organelles throughout evolution (Maul et al. 2002).

However, the fact remains that chloroplasts haven't fully transferred their genes to the nucleus throughout evolution. It is suggested that the potential toxicity of the ATP-generating electron transfer in the chloroplast is the selective pressure that retained the chloroplasts as separate organelles in the cell (Race et al.1999).

There is great variation between photosynthetic organisms in the number of chloroplasts per cell. Chloroplasts are polyploid and the number of chloroplast genome copies per organelle also varies a lot between different species. Up to date 45 chloroplast genomes have been sequenced (http://megasun.bch.umontreal.ca/ogmp/projects/other/cp_list.html), which represent a variety of taxonomical groups.

Structure and content of chloroplast genomes are uniform. In *angiosperms* and most algae, the genome is arranged with two inverted repeats (IRs) separating two single copy regions. Gene content is also highly conserved, but gene order varies.

Chloroplast genes generally show uniparental inheritance patterns, but examples are known of both paternal and maternal inheritance, so that no single mechanism is known for this phenomenon (Birky 1995).

1.2 *Chlamydomonas* as a model organism

Chlamydomonas is a unicellular alga found in a large variety of environments.

The genus *Chlamydomonas* was first described in the 19th century based on its main morphological features as seen by light microscopy – two flagella, a single large basal chloroplast containing one or more pyrenoids (the site of CO_2 fixation and the light-independent reactions of photosynthesis) and a cell wall (fig.1.2) (Harris 2001). The different species of *Chlamydomonas* were defined by variations of these morphological features.

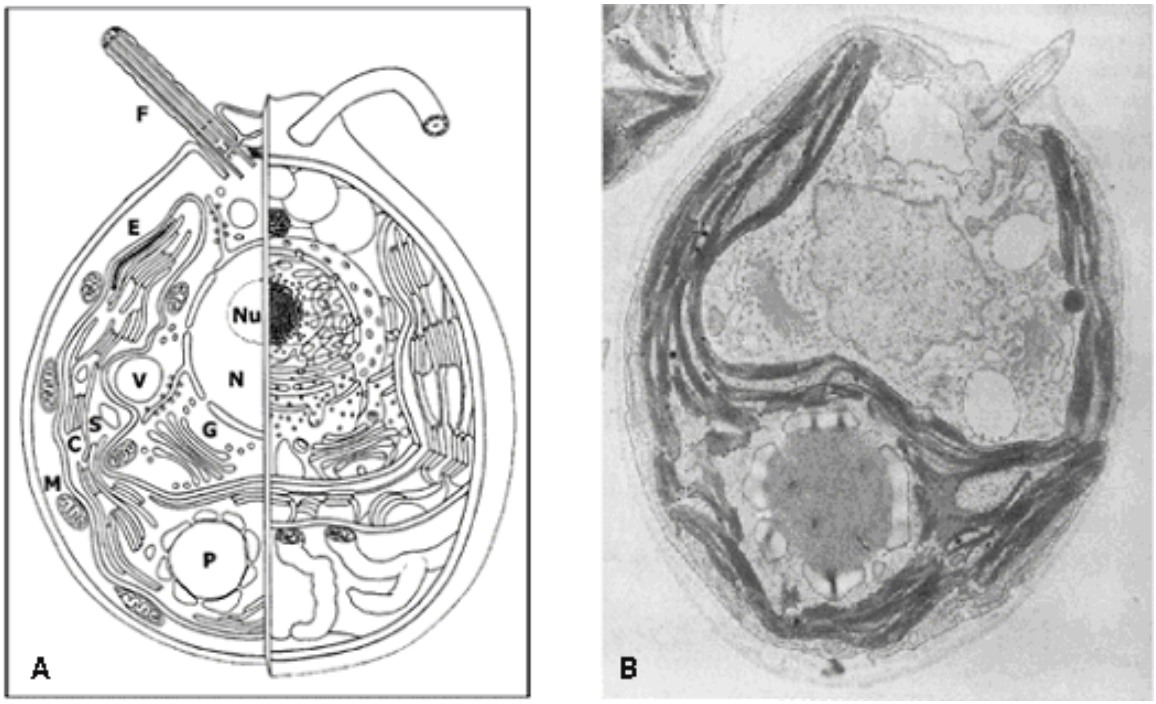


Fig 1.2 Chlamydomonas structure

A *C. reinhardtii scheme showing the central nucleus (N) with the nucleolus (Nu), the two isoform flagellae (F), the cup-shaped chloroplast (C) with the eyespot (E) and the starch containing pyrenoid (P) and the mitochondria (M). The golgi vesicle (G), starch grains (S), and the vacuoles (V). (Nickelsen & Kűck 2000)*
B *Electron micrograph (http://library.thinkquest.org/3564/Cells/cell92-1.gif)*

Chlamydomonas proved to be very suitable for genetic research (reviewed by Rochaix 1995; Lefebvre 1999; Harris 2001) and gained the name 'the green yeast'. The organism is easy and quick to grow, using simple medium. Its life cycle (fig 1.3) can be controlled by nitrogen and light. Nitrogen starvation triggers sexual propagation and light/dark cycles synchronize cell divisions. *Chlamydomonas* also provides simple follow-up possibilities, such as tetrad analysis, in which all four products of meiosis can be tracked, which was first discovered in 1918 by Pascher in this organism (Harris 1989). In addition, its growth in colonies on agar plates assists in follow up and isolation of cells with particular traits/genes.

An additional interesting trait of *Chlamydomonas* is that it is a facultative heterotroph. It is able to live either photosynthetically, or on a carbon source. This makes it ideal for photosynthesis research, since non-photosynthetic mutants are viable when grown in the presence of a carbon source other than CO_2.

Chlamydomonas contains three separate genetic systems – the nucleus, chloroplast, and mitochondria – which interact (reviewed by Rochaix 1995). The nucleus and organelles have different inheritance patterns. The nucleus displays mendelian inheritance, while the organelles display uniparental inheritance.

The organism is unique in that techniques have been found for the transformation of all three genomes, allowing extensive research on them (reviewed by Lefebvre 1999) and is widely used in the research of organelle biogenesis, photosynthesis, phototaxis, motility and more. It also provides additional insight on eukaryote cells in addition to the traditionally used model organism, the yeast.

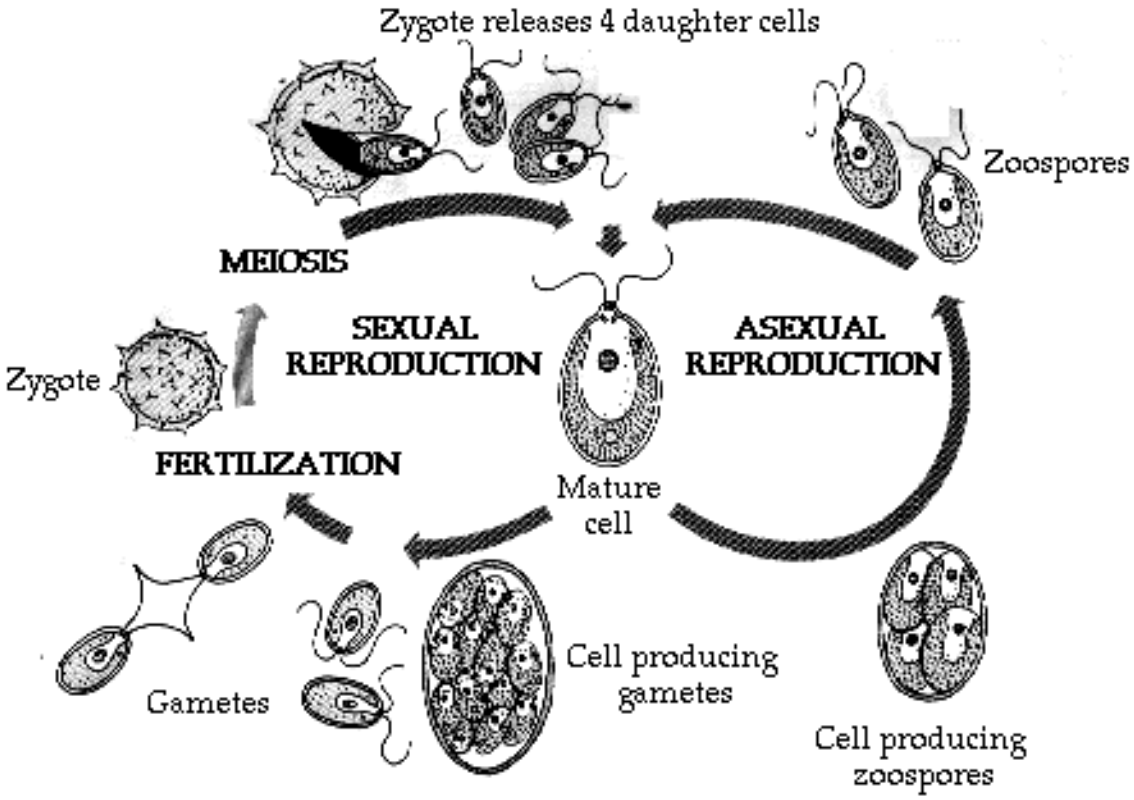


Fig 1.3 *Chlamydomonas life cycle (http://tidepool.st.usm.edu/crswr/chlamydomonaslc.html)*

1.2.1 *Chlamydomonas reinhardtii*

The most dominant *Chlamydomonas* species used in research today is *C. reinhardtii*, a strain was developed as a model organism in the 1940s and 1950s by Ralph A. Lewin and Ruth Sager. This strain can grow photosynthetically on simple salt medium, or grow in the dark if provided with acetate as a carbon source.
The three genomes of *C. reinhardtii* are sequenced and together with the available transformation techniques have made this organism ideal for detailed experiments.

1.2.1.1 The *C. reinhardtii* chloroplast

C. reinhardtii cells have a single chloroplast, which contains 50-80 copies of the genome. Like most chloroplast genomes, the chloroplast of *C. reinhardtii* contains two inverted repeats separating two single copy regions, but is unique in that its single copy regions are similar in size (Harris, 1989). It is generally accepted that the genome exists as a double stranded circular molecule, but experiments suggest that it may also exist in the form of dimers and in linear form (Maul et al. 2002).

The complete *C. reinhardtii* chloroplast genome sequence (fig 1.4) is now available (Maul et al. 2002). The genome is 203kb in size, distinctively larger than what is known for higher-plants model organisms such as spinach and tobacco, which are 150kb and 155kb in size, respectively (Maul et al. 2002). Its large size is the result of many changes it has accumulated and especially its many short repeats and introns (Boudreaux et al. 1994).

The genome codes for a complete set of tRNAs and has its own gene expression system (Maul et al. 2002). Interestingly, while chloroplasts are known to contain a nuclear-encoded RNA polymerase (NEP), such an enzyme has not been identified in *C. reinhardtii*, nor were the genes coding for it found in its nuclear genome (reviewed by Smith & Purton, 2002). However, promoters for both NEP and PEP are found in the chloroplast genome.

The chloroplast genes are organized in operon-like structures, but while this organization is conserved in land plants, gene placement in the algal genomes differs except in the IR regions, which as in higher plants, contain genes coding for ribosomal RNA (Rochaix, 1997).

Inheritance of the *Chlamydomonas* chloroplast genome is uniparental-maternal (mt+), though biparental inheritance is sometimes exhibited for some generations (Birky, 1995). The paternal chloroplast genome is degraded some time after zygote formation, but recombination between the maternal and paternal chloroplast genome can occur before degradation (Harris, 1989).

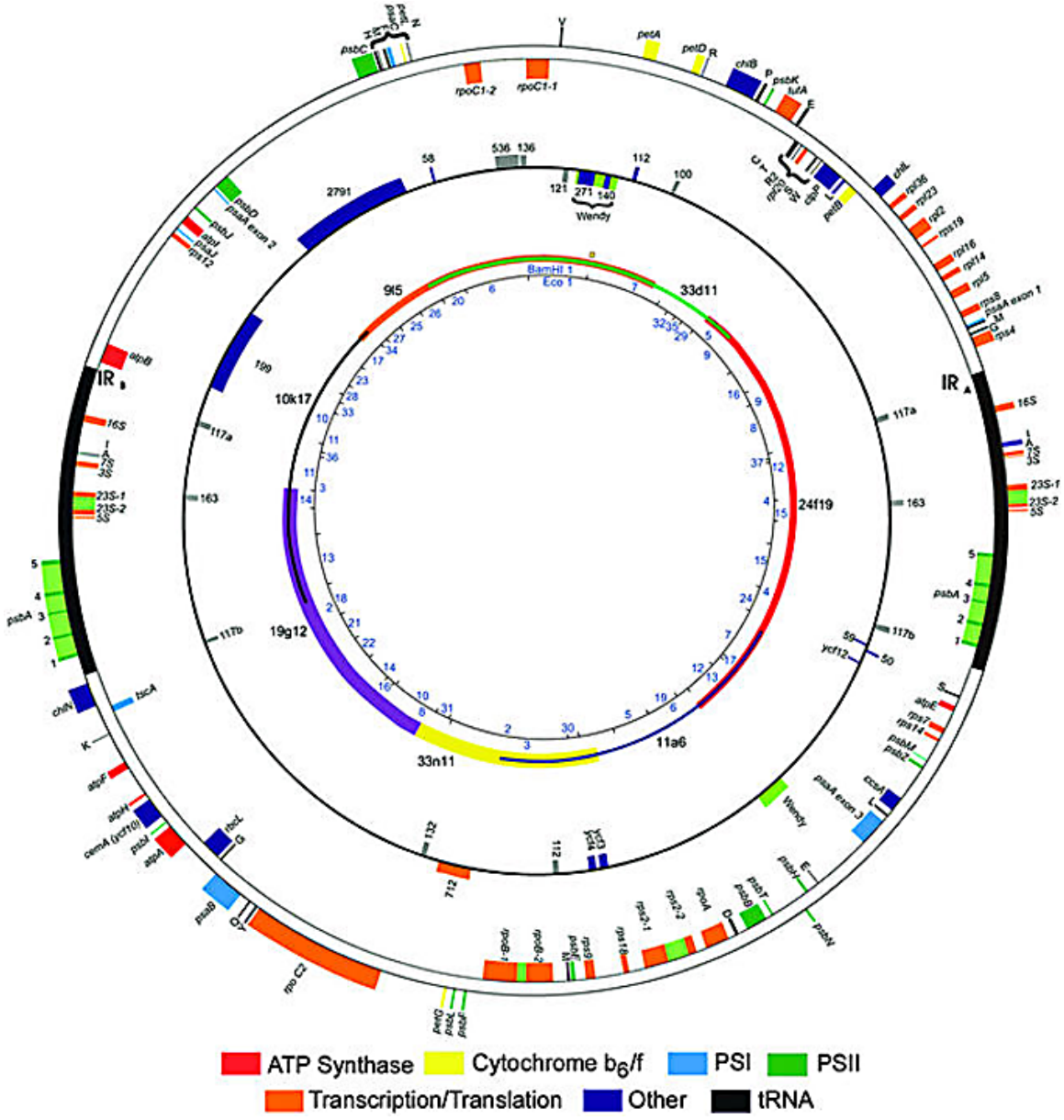


Fig 1.4 *C. reinhardtii chloroplast map (Maul et al. 2002)*

1.3 Chloroplast RNA processing & stability

In the chloroplast, just as in the nucleus, the process of gene expression is a cascade in which each phase is of importance and is regulated. RNA longevity is of major importance for gene expression. Transcription rate and the transcript stability are both involved in determining availability of transcripts for the translation machinery.

Chloroplasts have a rather constant pattern of relative transcription rates, and while it is regulated by environmental and developmental signals, it retains its relative transcription rates. This pattern is maintained despite transcription rate changes in response to environmental and developmental signals (Mayfield et al. 1995). The search for differential regulation of gene expression leads therefore further down the gene expression cascade to RNA stability and translatability, which have been shown to be determined by elements in the RNA molecules themselves.

RNA sequence and structure are crucial for its function since they interact with other elements active in the gene expression process. Messenger RNAs consist of a coding region surrounded by non-coding sequences called untranslated regions (UTRs). The 5'UTR and the 3'UTR play a major role in RNA stability, translatability and degradation. The structure of the untranslated regions depends largely on the maturation process of the RNA molecule. This section aims to present the maturation process of RNA in the chloroplast and its significance for gene expression in the chloroplast, as well as examine the final product, mature RNA, what defines its fate and how this affects gene expression.

1.3.1 RNA processing

Plastid RNA may undergo several types of processing before reaching its mature form, including RNA editing, RNA splicing, intercistronic-endonucleolytic cleavage, 5'-end maturation and 3'-end maturation. The results of this processing affect the stability, translatability and degradation of mature RNA. In many cases, specific nuclear loci are found that are involved in chloroplast RNA processing, suggesting potential differential regulation of chloroplast gene expression.

1.3.1.1 RNA editing

RNA editing is a process in which individual nucleotides in the sequence of a transcript are changed and no longer coincide with the genomic sequence from which they were transcribed. This process was found in the chloroplast mRNA of land plants (Smith et al. 1997), but not of *cyanobacteria* and algae (Bock 2000).

The most common editing event in chloroplasts is the conversion of a genomic-encoded C residue into a U residue, though examples for the opposite conversion (U→C), also called 'reverse editing', have also been found (Yoshinaga et al. 1996). While some editing events are silent ones, many are known to have functional significance. Editing sites have been found to create initiation (ACG→AUG) and termination (CGA→UGA) codons and are also involved in restoring conserved amino-acids in proteins and conserved elements in untranslated regions of RNAs (Hoch et al. 1991; Yoshinaga et al. 1996; Maier et al. 1992; Kudla & Bock, 1999; Maier et al. 1995).

The mechanism in which editing occurs in the chloroplast appears to be a base conversion mechanism that involves both *cis*-acting elements flanking the editing sites and site-specific *trans*-acting factors (Bock 2000). No consensus in sequence or secondary structure is known for the *cis*-acting elements, and none of the *trans*-acting factors involved in this conversion have been identified, but some chloroplast-encoded

site-specific RNA-binding proteins have been found to interact with the *cis*-elements (Hirose & Sugiura 2001; Miyamoto et al. 2002).

Editing appears to occur early in RNA processing, prior to other RNA processing events like splicing and intercistronic cleavage (Freyer et al. 1993; Ruf et al. 1994). At the same time, evidence for the dependence of editing on translation is varying. While many editing sites show complete independence from translation (Zeltz et al. 1993), others exhibit complete or partial dependence of editing efficiency on translation (Karcher et al. 1998). This, however, is likely to be the result of a possible effect of ribosome-binding on secondary structures that may be obscuring editing sites from *trans*-acting factors, and does not contradict the early occurrence of editing in the processing of mRNA.

As with other stages in chloroplast gene expression, RNA editing and also responds to environmental and developmental signals and changes in editing efficiency in response to such signals have been observed (Bock et al. 1993; Ruf & Kössel 1997; Karcher 1998; Hirose & Sugiara 1997; Karcher & bock 2002).
In light of its role in transcript translatability, and the evidence of non-functional proteins and mutant phenotype resulting from translation of unedited transcripts (Bock et al. 1994; Zito et al. 1997, Sasaki et al. 1997), it can be assumed that editing has a functional role in the regulation of gene expression in the chloroplast. However, differential editing in response to various cues has not been proven. Recent evidence shows that other parameters, such as transcript abundance, play a more significant role than editing in adapting gene expression in the chloroplast according to developmental signals. This points towards a mutation-correction role for editing, rather than a role in regulating gene expression (Peeters & Hanson 2002).

1.3.1.2 Intron splicing

A number of chloroplast genes, including rRNA-, tRNA- and mRNA-coding genes, contain introns, which must be spliced during the maturation process of the RNA molecule.

Splicing can connect between different exons in the same transcript ('*cis*-splicing'), or between exons on different transcripts ('*trans*-splicing').

Plastid introns are divided into three major classes known as Groups I, II and III, based on their structural elements and splicing fashion. Organelle introns differ from nuclear introns in structure and function, although similarities are found between group II plastid introns and nuclear introns. It is suggested that organelle introns may have been the origin of nuclear introns (Saldanha et al. 1993). At least some introns of groups I and II exhibit self-splicing in vitro. However, it is likely that even self-splicing introns require *trans*-acting factors for their splicing in vivo (Lambowitz & Perlman 1990).

Nuclear loci, chloroplasts RNAs and chloroplast proteins are known to be involved in intron splicing in the chloroplast (Jenkins et al. 1997; Hubschmann et al. 1996; Hess et al. 1994).

There is evidence for the regulation of splicing by developmental and environmental cues. Some group II introns in land plants exhibit tissue-specific differences in their splicing (Barkan 1989), and the splicing of several group I introns in *C. reinhardtii* is stimulated by light, via photosynthetic electron transport (Deshpande et al. 1997).

In addition, functional significance of splicing has been found, as in the case of the *Chlamydomonas psb*A transcripts, in which splicing efficiency is linked to the organism's ability to grow photosynthetically (Lee & Herrin 2003). It therefore seems that intron splicing is of importance in chloroplast gene expression.

1.3.1.3 Intercistronic processing

Many plastid genes are organized in operons and transcribed as polycistronic RNAs, which are then cleaved into monocistronic transcripts. Intercistronic cleavage is of importance for gene expression in chloroplasts, since its absence can prevent the translation of proteins encoded by the polycistronic transcript.

Several known examples show different ways in which intercistronic processing can affect chloroplast gene expression.
The translation of *psa*C and *ndh*D in tobacco depends on the cleavage of the dicistronic *psa*C-*ndh*D transcript because in its dicistronic form, a base pairing between an element in the coding region of *psa*C and an element in the 5'UTR of *ndh*D forms a structure that inhibits ribosome access to the initiation codons of both *psa*C and *ndh*D (Hirose & Sugiura 1997). A similar structural barrier occurs in the dicistronic transcript of *pet*D and *pet*B in maize, but in this case a site-specific nuclear-encoded factor, crp1, allows the cleavage and translation of the transcripts (Barkan et al. 1994). The involvement of nuclear gene products in intercistronic cleavage opens up a possibility for differential regulation of gene expression through this type pf processing.
Examples for intercistronic cleavage of rRNA (Barkan 1993; Holloway & Herrin 1998) show how this type of RNA processing can control the availability of the translation machinery. In these examples nuclear loci are responsible for this processing, which allows ribosome assembly.
In addition, intercistronic processing can affect chloroplast gene expression is through its role in the maturation of the 3'UTR and 5'UTR (sections 1.3.1.4, 1.3.1.5), which contain stability and instability elements (section 1.3.2).

1.3.1.4 5'end maturation

The maturation of chloroplast transcripts requires in some cases 5'end processing. Two types of processing are known – endoribonuclease cleavage and 5'→3' exonuclease trimming.

Ribonucleolytic cleavage is often observed by the finding of two transcript populations for the same gene, one with a 5'end corresponding to transcription initiation site and the other shorter and with a 5'end corresponding to a processing site. Studies show a correlation between transcript processing and translation. In *Chlamydomonas*, the processed transcript was found to be the only translatable transcript form (Nickelsen et al. 1999; Bruick & Mayfield 1998; Vaistij et al. 2000).

Ribonucleolytic cleavage may be followed by 5'→3' exonuclease trimming (Drager et al. 1998). Exonuclease trimming may also follow intercistronic cleavage, which makes the 5'end of a transcript available for it.

Nuclear-encoded factors are involved in the 5'end processing of transcripts and are important for the translatability of the transcripts (Vaistij et al. 2000; Nickelsen et al. 1999). It has also been established that 5'end maturation responds to environmental signals (Shapira et al. 1997; Reinbothe et al. 1993).

1.3.1.5 3'end maturation

The 3'UTRs of chloroplast mRNAs usually contain inverted repeats (IRs) that can form stem-loop structures, which stabilize the transcript (Stern & Gruissem 1987; Stern et al. 1989). These IRs protect from transcript degradation by preventing exonuclease progress, or by binding proteins that protect from degradation (Stern & Gruissem 1987; Stern et al. 1989).

Transcription of chloroplast genes is not efficiently terminated by the 3'UTR (Rott et al. 1996). The process of 3'end maturation is made of endonucleolytic cleavage downstream of the IRs, followed by 3'→5' exonuclease trimming until the first encountered IR stem (Stern & Kindle, 1993; Hayes et al. 1996). However, it seems that the endonucleolytic cleavage site is not always necessary for 3'end maturation (Rott et al. 1999; Stern et al. 1991) and that alternative maturation pathways may exist, as in the case of 3'ends generated through the cleavage of polycistronic transcripts.

A number of proteins, mostly nuclear-encoded, are involved in 3'end processing (reviewed by Monde et al. 2000).

As with 5'end maturation, 3'end maturation appears to modulate translatability of chloroplast mRNAs and promote polysome association (Rott et al. 1998).

1.3.2 RNA stability

As mentioned before, RNA is characterized by both sequence and secondary structure. Intrinsic elements of both sequence and secondary structure have been found that affect transcript stability.

1.3.2.1 5'UTR mRNA stability elements

RNA stability elements have been found in the chloroplasts of higher plants (Shiina et al. 1998) and of algae (Higgs et al. 1999; Anthonisen et al. 2001).

Extensive research on the role of 5'UTR in chloroplast mRNA has been done in *Chlamydomonas*. 5'UTR stability elements have been well defined in the *rbc*L and *atp*B genes of *Chlamydomonas* (Anthonisen et al. 2001). In addition, examples of nuclear mutations have been found that affect mRNA stability through the interaction of the nuclear gene products with the 5'UTRs of chloroplast mRNAs (Vaistij et al. 2000; Nickelsen et al. 1994; Drager et al. 1998; Esposito et al. 2001).

Interestingly, the 5'UTRs of different genes provide different degrees of stability to transcripts (Eibl et al. 1999). Transcript stability has been shown to compensate for changes in transcription rate in response to environmental stimuli (Shiina et al. 1998; Salvador et al. 1993) and transcripts coding for products with different roles may need to be stabilized to a different degree under different conditions.

1.3.2.2 5'UTR mRNA instability elements

While in most cases elements in the 5'UTRs are found to confer transcript stability, some examples of the opposite effect have been found. Such examples are the AU-motif of cyanobacterial *psb*A2 5'UTR, which confers instability in the dark (Agrawal et al. 2001) and the +21 to +41 sequence of *Chlamydomonas rbc*L 5'UTR, which is required for photo-accelerated degradation (Singh et al. 2001).

1.3.2.3 3'UTR mRNA stability elements

As mentioned above (section 1.3.1.5), most chloroplast 3'UTRs contain inverted repeats (IRs) that fold into stem-loops. The IRs are known in some cases to be transcript-stabilizing (Stern et al. 1987, 1989,1991; Lee et al. 1996; Monde et al. 2000; Rott et al. 1998). The IRs function as a protection from 3'→5' exoribonuclease (Drager et al. 1996). Some examples of orientation-dependent IR function (Rott et al. 1998; Blowers et al. 1993) suggest that the orientation may determine the formation of 3'ends or the binding of proteins, which prevent ribonuclease degradation (Rott et al. 1998). Site-specific RNA-binding proteins that bind to 3'UTRs have been identified (Memon et al. 1996; Levy et al. 1999).

At the same time, there is also evidence of IRs being unnecessary for mRNA stabilization (Blowers et al. 1993). Moreover, the IR sequence itself does not appear to be required for mRNA stabilizing (Drager et al. 1996). The 3'IRs sequences of the *rbc*L gene of non-flowering land plants and algae have not been found to be conserved (Calie & Manhart 1994).

1.3.2.4 mRNA stability/instability elements in the coding region

Stability determinants have been found also inside the coding region of mRNAs (Kulkarni & Golden 1997; Singh et al. 2001; Drapier et al. 2002). These were found to function in different ways, such as stalling ribosomes (Kulkarni & Golden 1997) and interacting with determinants in the 5'UTR, possibly blocking endonuclease attack (Singh et al. 2001).

Instability determinants have been found in the coding region of spinach *psb*A mRNA, where degradation that is initiated by endonucleolytic cleavage in the coding region is probably directed by structural sequences at the cleavage sites (Klaff 1995). The cleavage of these sites is regulated by magnesium ions, which block the site and stabilize the mRNA (Horlitz & Klaff 2000).

1.3.3 Regulation of mRNA stability

1.3.3.1 Developmental and environmental signals

Transcription rate fluctuations do not account for differential changes in mRNA levels (Mayfield et al. 1995). Experiments with transcription inhibitors point out RNA stability as a differential regulator of RNA levels, responding to developmental and environmental conditions (Klaff & Gruissem 1991; Kim et al. 1993).

Interestingly, in *Chlamydomonas*, increased mRNA stability has been found to compensate for decreased transcription rates in the dark, keeping RNA levels steady (Salvador et al. 1993). Similar examples for such compensation have also been reported in tobacco and barley (Shiina et al. 1996; Kim et al. 1993).

Evidence from *Chlamydomonas* points out redox-carriers in the chloroplast as potential transmitters of light stimuli to the stabilization apparatus of chloroplast transcripts (Salvador & Klein, 1999).

The interaction of nuclear gene products with stability elements (sections 1.3.2.1 and 1.3.2.3) is likely to be a transmission pathway of developmental signals to the chloroplast.

1.3.3.2 Trans-regulatory factors

RNA-binding proteins are abundant in the chloroplast stroma (Nakamura et al. 1999) and are found associated to most ribosome-free stromal mRNAs (Nakamura et al. 2001). It is proposed that proteins bind to mRNAs immediately after transcription and both stabilize the transcript by reducing ribonuclease access, as well as promote RNA maturation processes (Nakamura et al. 2001).

Association to RNAs may be regulated by phosphorylation and redox potential in response to light stimuli (Danon & Mayfield, 1994; Kim & Mayfield 1997; Liere & Link 1997; Lisitsky & Schuster 1995).

Non-proteinaceous trans-acting factors have also been found to regulate RNA stability. Magnesium ions affect chloroplast RNA stability in both *Chlamydomonas* and plants (Klaff, 1995; Nickelsen et al., 1994). In spinach, different RNAs obtain stability at different magnesium concentrations (Klaff, 1995). It is also observed that the concentration of free magnesium ions rises during chloroplast development to a level that can mediate RNA stabilization (Horlitz & Klaff, 2000). It is suggested that magnesium ions may prevent RNA degradation through protecting endonucleolytic cleavage sites and through influencing protein binding to the mRNA UTRs.

Other non-protein trans-acting factors may very well exist. Phosphate is such a candidate for the regulation of chloroplast mRNA stability, but its involvement has not yet been proved (Bollenbach et al. 2004).

1.3.3.3 Ribosome association

While some examples in higher plants show ribosome association to transcripts to be destabilizing for mRNAs (Klaff & Gruissem 1991), other examples, in maize (Barkan 1993) and *Chlamydomonas* (Yohn et al. 1996; Bruick & Mayfield 1998), show the opposite effect of ribosome binding.

1.3.4 RNA degradation

Chloroplast RNA longevity varies a lot between different transcripts. The degradation mechanism of chloroplast mRNA consists of endonucleolytic cleavage followed by pholyadenylation and 3'→5' endonuclease or 5'→3' exonuclease degradation (reviewed by Hayes et al. 1999; Schuster et al. 1999; Bollenbach et al. 2004).

As mentioned in above, chloroplast RNA degradation may be affected by factors regulating cleavage sites accessibility, such as magnesium ions, RNA-binding proteins and ribosome association. Chloroplast RNA degradation may also be affected by the length and composition (Guanosine content) of the polyadenylated tail (Monde et al. 2000).

1.4 Chloroplast transformation

A revolutionary method for plant cells transformation was reported in 1987 (Klein et al. 1987). Using microprojectiles shot at high velocity (fig. 1.5) nucleic acids could be delivered into living cells. In 1988, it was reported that the method, also referred to as 'biolistics', could be used to transform organelles (Boynton et al. 1988). Due to a homologous recombination mechanism in the organelles, stable transformation was accomplished when using DNA with sequences homologous to organelle genome sequences. Foreign DNA could also be incorporated into an organelle genome if flanked by homologous sequences (Blowers et al. 1989).

Mutation complementation is commonly used for transformant screening. In *Chlamydomonas*, a working method was developed for the selection of transformants using a non-photosynthetic mutant *Chlamydomonas* strain for the transformation and complementing its mutation with the inserted DNA, using restored photosynthetic-ability as a selection marker for transformants (Blowers et al. 1989).

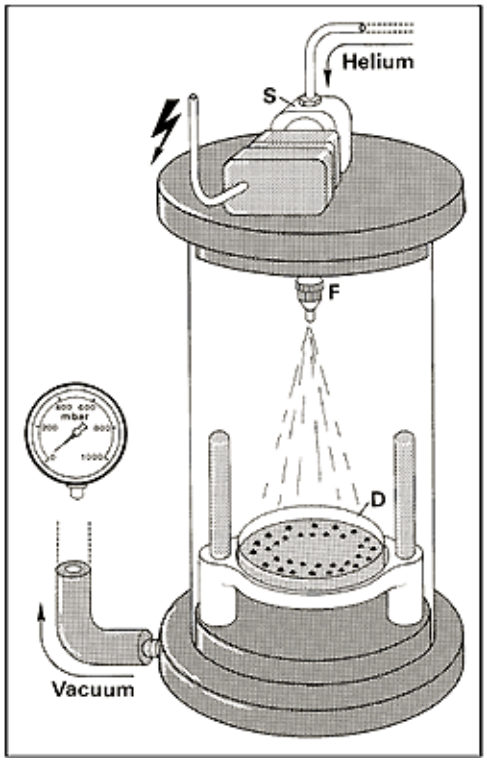


Fig. 1.5 *Illustration of a particle gun. Helium pressure is released into the reaction chamber via a syringe filter (F) carrying the DNA coated microprojectiles. Helium release is controlled by a timer relay-driven solenoid (S), which opens the valve for 50 ms. The accelerated particles hit the algal cells, which are spread on the surface of a petri dish (D) on the bottom of the reaction chamber (Nickelsen & Kűck 2000).*

1.5 The scope of this study

The *rbc*L gene is a chloroplast gene that codes for the large subunit of ribulose-1.5-biphosphate carboxylase/oxygenase (RUBISCO). This enzyme is a part of the photosynthesis apparatus and is responsible for CO_2 fixation.

RNA secondary structure is of importance for RNA stability (section 1.3). The *Chlamydomonas rbc*L 5'UTR has been extensively studied for its role in *rbc*L transcript stability. The location of a restriction enzyme site that allows cutting the transcribed sequence from the DNA exactly at +1 (transcription start site) position makes this gene particularly ideal for 5'UTR research.

The secondary structure of the *Chlamydomonas rbc*L 5'UTR (fig 4.1, discussion) has been deduced and is predicted to consist of a large stem-loop (nucleotides +1 to +41) followed by a smaller stem-loop (nucleotides +49 to +63) downstream of it (Anthonisen et al. 2001, Singh et al. 2001).

In this work, the importance of the sequence of the large stem-loop is examined by introducing two different changes to it. Work on the *rbc*L 5'UTR was done with constructs made of the changed *rbc*L 5'UTR, followed by the *E. coli uid*A gene and *Chlamydomonas psa*B 3'region downstream of it. The constructs were incorporated into the chloroplast genome between the *atp*B gene and the IR (fig 2.1, materials and methods).

The first change tested reverses the nucleotide sequence in positions +5 to +37, while the second change examined is a complete change of sequence, in which nucleotides +6 to +36 are changed so that each purine is replaced by another purine (A→G; G→A), and each pyrimidine by another pyrimidine (C→T; T→C) (fig 4.3, discussion). Both of these introduced changes affect only the sequence of the predicted large stem-loop, but not its structure.

2. MATERIALS AND METHODS

2.1 Strains and Media

2.1.1 *Escherichia coli*

Recombination deficient strain TB1 was used for cloning.

Cultures were grown overnight at 37°C, either in LB medium using a shaker/rotator, or on LA petri dishes (LB medium + 15g agar per liter).

Long-term storage of cells was done in a 15% glycerol solution at -80°C.

2.1.1.1 Preparation of competent cells for transformation

Cells were $CaCl_2$-treated (Sambrook and Russell 2001).

2.1.1.2 Transformation

Heat-shock treatment was used (Sambrook and Russell 2001).

Transformants (ampicillin-resistant) were selected using ampicillin-containing LA petri dishes.

2.1.1.3 Plasmid isolation

Selected transformants were grown overnight on a rotating wheel in LB medium containing ampicillin (50μg/ml).

For further use in cloning, a small-scale plasmid isolation (miniprep) was used (Sambrook and Russell 2001).

For transformation of *Chlamydomonas*, DNA was isolated by large-scale plasmid isolation (maxi-prep) (Sambrook and Russell 2001).

2.1.2 *Chlamydomonas reinhardtii*

2.1.2.1 Strains

The mutant strain CC-373 (ac-u-c-2-21) was obtained from the *Chlamydomonas* Genetics Center at Duke University, North Carolina. The strain is light-sensitive due to a deletion of part of its *atpB* gene (fig 2.1), which codes for a part of the chloroplast ATP synthetase. It is heterotrophic, and can be grown in the dark using acetate as its only carbon source.

The strain is used for transformation, using restored photosynthetic capability as a means of selection (Blowers et al. 1989).

MU7 (Salvador et al. 1993a) contains an unmodified version of the *rbc*L-GUS construct (fig 2.1) and was used for comparison of RNA abundance with the CGstem and 5'reverse transformants tested in this work.

2.1.2.2 Growth conditions

CC-373

HSHA medium – a high salt (HS) medium (Sueoka 1960) enriched with potassium acetate (2.5 g/l).

Grown in the dark at room temperature.

MU7 and transformed CC-373

HS medium (Sueoka 1960).

Growth in water bath at ~30°C, under continuous mixing by air and 2% CO_2, either in constant light, or in 12 hours light/12 hours dark cycles.

2.1.2.3 Preparation of cells for transformation

CC-373 cells were grown for several days, supplied with fresh medium daily. Cells were grown in the dark, at room temperature, on a shaker. Cells were ready for transformation when reaching growth log phase.

2.1.2.4 Transformation

The biolistic particle delivery system (PDS-1000/He; BioRad) was used according to BioRad's operation protocol (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9075.pdf, section 4) with 0.6 μm gold particles as microcarriers. The system's parameters were adjusted and the cells were prepared as described by Boynton *et al.* (1988).

2.1.2.5 DNA isolation

As described by Dellaporta et al. (1983).

2.1.2.6 RNA isolation

RNA was isolated from cultures grown in light/dark cycles after 11 hours in the dark and after 1 hour in the light, using the SDS/phenol method (Merchant and Bogorad 1986).

2.2 Plasmids

p+157SK+ (Anthonisen et al. 2001):
A ~5kb plasmid, based on pBluescript SK+ (Stratagene) with a ~2.2kb fragment cloned between its *Xho*I and *Xba*I sites of its polylinker. The 2.2kb fragment contains 227bp that originate from positions –70 to +157 (transcription start site being +1) of the *C.reinhardtii rbc*L gene. These 227bp contain the *rbc*L promoter, its 5'UTR and some of the *rbc*L coding sequence. The 2.2kb fragment (fig 2.1) is made of these 227bp fused 5' of the coding region of the *E. coli uid*A gene coding for β-glucuronidase (GUS). The plasmid contains an ampicillin resistance gene for selection in transformed bacteria.

pCrc32 (Blowers et al. 1993): An ~11kb plasmid, used for *Chlamydomonas* transformation. It contains an ampicillin resistance gene for selection in transformed *E. coli*, as well as sequences homologous to *C. reinhardtii* chloroplast DNA (cpDNA), which promotes homologous recombination with the cpDNA during transformation. These sequences complement the *atp*B-IR deletion found in the *Chlamydomonas* strain CC-373 (fig 2.1).

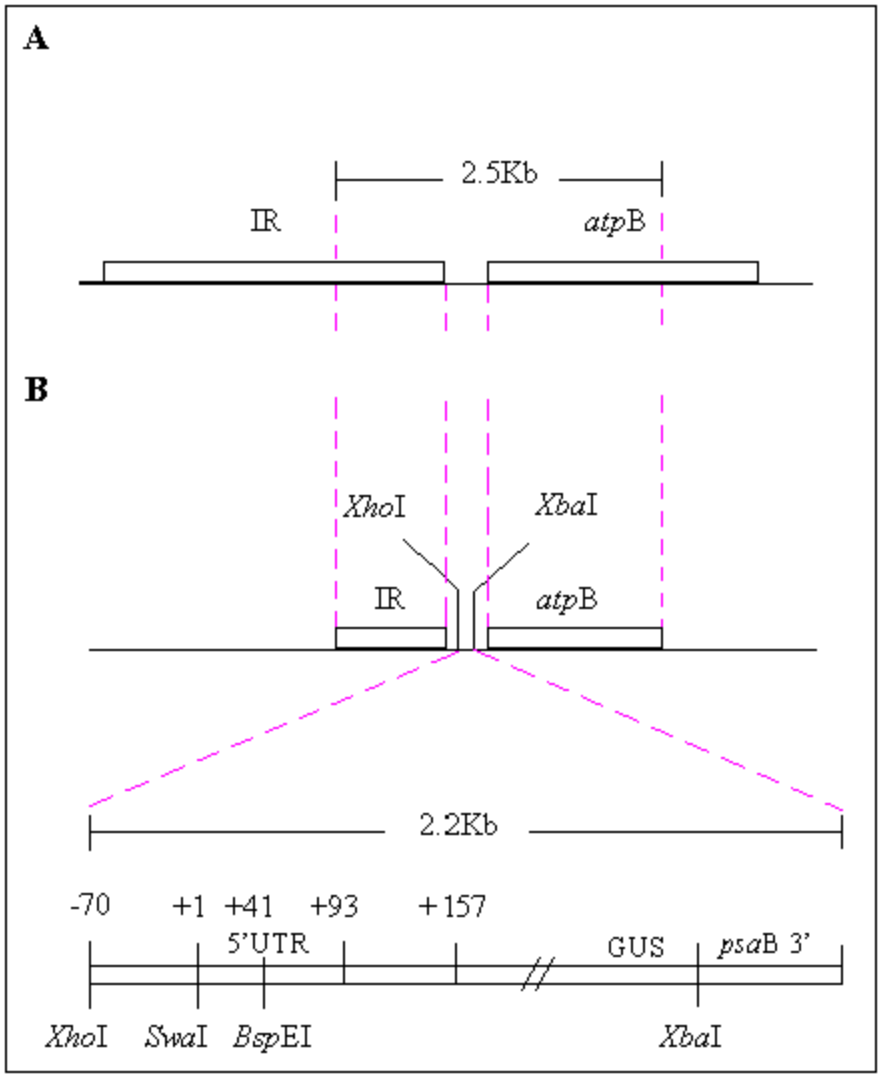


Fig 2.1 **A** *The 2.5kb deleted region in cc-373.* **B** *The transformation vector with homologous sequences complementing the deletion in cc-373 and carrying the 2.2kb construct made of rbcL 5'UTR, uidA (GUS) and psaB 3' region.*

2.3 DNA manipulation methods

2.3.1 Oligonucleotide annealing

Synthetic oligonucleotides, phosphorylated at their 5'-end, were obtained from MWG Biotech AG (fig 3.1, results).
The annealing was done by mixing equal amounts of the oligonucleotides and heating the mixture to 100°C for two minutes, followed by cooling down to room temperature for about 15 minutes (Sambrook and Russell 2001).
Concentration of the resulting dsDNA fragments was determined by calculation from the oligonucleotide data, supplied by MWG Biotech AG.

2.3.2 Restriction enzymes

Restriction enzymes from New England Biolabs and Promega were used according to producer instructions.

2.3.3 Gel electrophoresis

A 1% agarose gel containing ethidium bromide was used in TAE buffer. Gel loading buffer was added to samples (2% of total sample volume).
A 1Kb plus ladder (Invitrogen) (fig 2.2) was used to estimate the size of DNA fragments.

2.3.4 Isolation of DNA fragments by gel electrophoresis

DNA fragments were separated on an agarose gel and isolated from it for further use in cloning. The agarose gel was prepared for the isolation by cutting a "well" in it, right below the DNA band and inserting a dialysis-membrane into it to seal it (fig 2.3).
The gel was then put back into the electrophoresis chamber, this time with enough TAE buffer to cover its sides, but not flow over the upper surface of the gel. The well that was made was filled with TAE buffer and electrophoresis was resumed until the DNA band reached the TAE buffer that filled the well. The TAE buffer in the well was collected using a pipette and the DNA was purified by phenol extraction and ethanol precipitation.

2.3.5 Ligation

T4 DNA ligase was used according to the protocol (Sambrook and Russell 2001).
For optimized ligation, total ligation mixture volume was 10µl and vector-insert ratio was determined as follows:

- The amount of vector used was determined by its size: ~ 66.5ng per 1kb.
- The amount of insert was determined as followed – 1.3 times the amount of the vector (in moles) for inserts of 0.5-3kb, and 5 times the amount of the vector (in moles) for inserts smaller than 100bp.

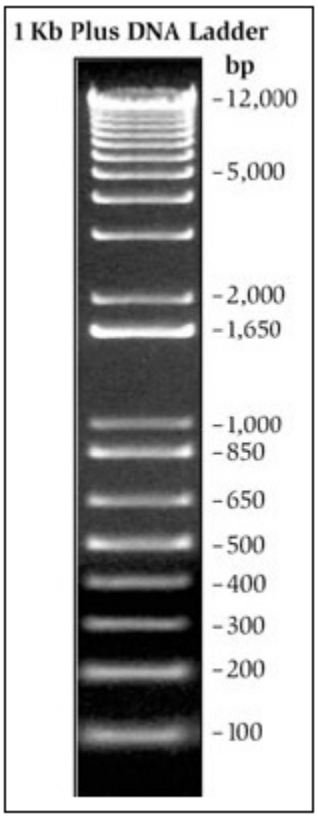


Fig 2.2 *1Kb+ ladder, Invitrogen*

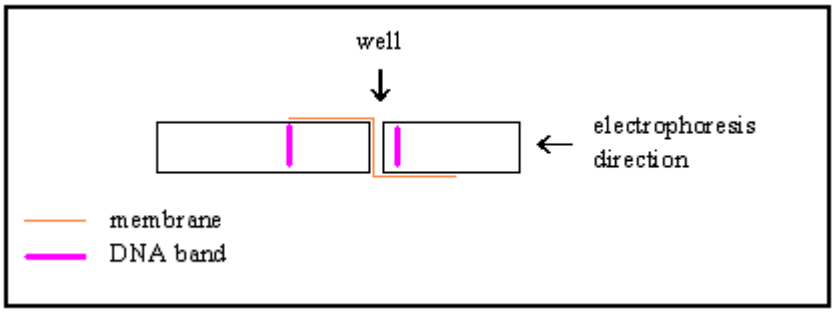


Fig 2.3 *Isolation of DNA fragments by gel electrophoresis*

2.4 DNA Sequencing

Cloning results were verified by sequencing the region of the resulting plasmid that should contain the new insert. Automatic sequencing was performed with a Megabace machine (GE Healthcare) based on the chain termination method (Sanger et al. 1977) using a GUS primer.

2.5 DNA/RNA quantification

Estimation of DNA concentrations was done by dot spot analysis (Sambrook and Russell 2001).
More accurate measurements of DNA and RNA concentrations were used when analyzing *Chlamydomonas* transformants and were performed by spectrophotometer at 260nm (Sambrook and Russell 2001).

2.6 Hybridization analysis methods

2.6.1 Probes

Probes used for hybridization analyses were labelled with α-^{32}P-dCTP by random primer labeling using the Klenow fragment of *E. coli* DNA polymerase I (Sambrook and Russell, 2001).
GUS (~1.9kb) and *atp*B (~700bp) templates for random primer labeling were obtained from plasmid pBI221 (Jefferson 1987) by digestion with *Bam*HI and *Sac*I and from plasmid pCrc*atp*B (Blowers et al. 1990) by digestion with *Bam*HI, respectively.

2.6.2 DNA slot/dot blot

1µg genomic DNA samples were denatured with NaOH, applied to the assembled slot blot apparatus (PR600, Hoefer Scientific Instruments) and transferred to a ZetaProbe membrane (BioRad) (Sambrook & Russel 2001). Further fixation of the DNA to the membrane was done using UV crosslinking (254nm, 3 minutes).

The membrane was hybridized with the radiolabeled GUS probe (section 2.6.1). Hybridization and washing were done according to the BioRad protocol (http://www.bio-rad.com/cmc_upload/Literature/12643/LIT234C.pdf , section 4).

Kodak Biomax MS film was exposed to the membrane at -80°C. Exposure time depended on the radioactivity level of the probe (- the lower the radioactivity level, the longer exposure time). The film was developed using Kodak film developer.

2.6.3 Southern blot

1.5µg genomic DNA was digested with *Hind*III and *Kpn*I and run on a 1% agarose gel. The DNA was transferred from the gel to a ZetaProbe membrane (BioRad) by alkaline capillary transfer according to the ZetaProbe protocol (http://www.bio-rad.com/cmc_upload/Literature/12643/LIT234C.pdf , section 2.1) and UV crosslinked as described in section 2.6.2.

The membrane was hybridized to the *atp*B probe (section 2.6.1). Hybridization, washing and exposure to film as described in section 2.6.2.

2.6.4 Northern blot

RNA samples were denatured by dissolving in formamide/formaldehyde solution and heating for 15 minutes at 65°C. 4µg RNA of each prepared sample was applied to and separated on a 1.3% agarose/formaldehyde gel (Sambrook and Russell 2001). RNA was transferred to a ZetaProbe membrane (BioRad) by alkaline capillary transfer according to the ZetaProbe protocol (http://www.bio-rad.com/cmc_upload/Literature/12643/LIT234C.pdf, section 2.2).

The membrane was hybridized to the GUS probe (section 2.6.1). Hybridization, washing and exposure to film as described in section 2.6.2.

3. RESULTS

3.1 The CGstem construct

3.1.1 CG-Stem construct cloning

3.1.1.1 Cloning CGstem into the pBluescript SK+ plasmid

Single-stranded oligonucleotides (fig 3.1) were hybridized to form a double-stranded DNA insert. The DNA insert formed had one blunt end and one sticky end that complement the 5' end formed by *Swa*I restriction and the 3' overhang formed from *Bsp*EI restriction, respectively (fig 3.1).

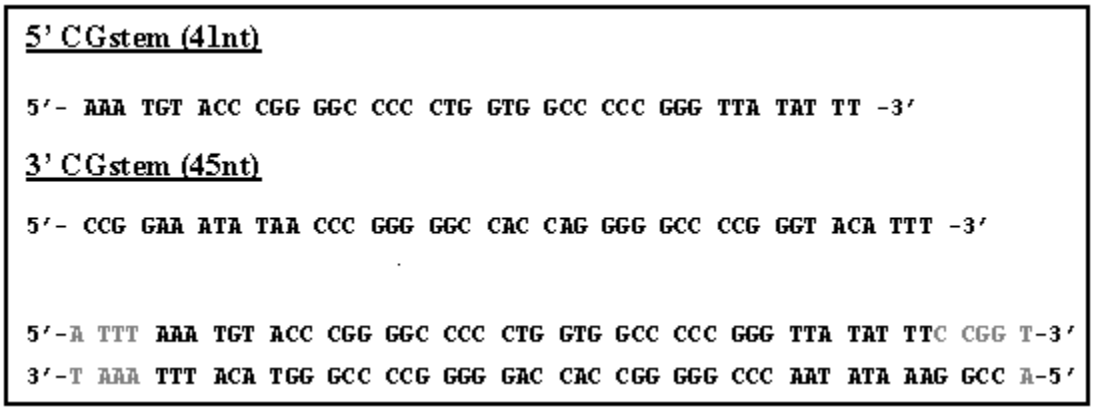


Fig. 3.1 The CGstem oligonucleotides and the double-stranded insert formed after annealing. *The insert's ends match those formed by cleavage with BspEI and SwaI. Nucleotides marked in gray show the completing parts of the BspEI and SwaI restriction site sequences, but are not part of the ligated DNA insert.*

The plasmid p+157SK+ was cut with *Swa*I and *Bsp*EI and agarose gel electrophoresis was used to separate the resulting fragments and isolate the larger one (~5kb) gel (fig 3.2). Concentration of the isolated plasmid fragment was determined by dot spot analysis.

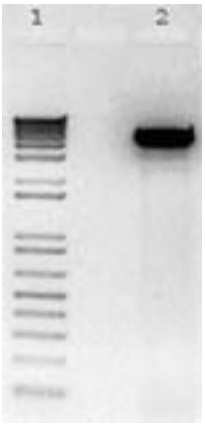


Fig 3.2 p+157SK+ cutting

1) 1Kb+ ladder.

2) p+157SK+ cut with SwaI and BspEI.

No control for the efficiency of this double digestion was possible at this stage, since the released fragment was too small to be visible on the gel (41bp).

The isolated plasmid fragment and the insert (the hybridized oligonucleotides) were ligated. It was expected that if the p+157SK+ plasmid was successfully double cut, religation of the plasmid will not occur in the absence of the insert. A parallel ligation was therefore done in the absence of the insert, in which the insert was replaced with water to maintain equal reaction volume. If the plasmid relegated in the absence of the insert it would then be visible when attempting transformation of competent *E.coli* cells with these ligation mixes, since the transformation is only possible with intact circular DNA.

Transformation was done with the two ligation mixes as well as with the original p+SK+157, which served as control for transformation efficiency. The cells were spread on LB-ampicillin agar plates for transformant selection.

The transformation with p+157SK+ resulted in many colonies, proving the transformation procedure was successful and the cells were competent.

Only three colonies resulted from the transformation with the ligation mix that lacked the insert, while about 150 colonies arose from the standard ligation mix. This proved that most of the p+157SK+ was successfully double-cut and not just religated without incorporating the new insert.

A miniprep on transformant colonies assumed to carry the cloned plasmid (fig 3.3) showed a plasmid of expected size (~5kb) present in transformants colonies that were randomly picked, reassuring no false-positives from naturally ampicillin-resistant *E.coli* colonies.

A maxi-prep was performed on two of the selected transformants and used for sequencing. The sequencing was successful (97% overall quality) and verified the incorporation of the insert into the plasmid.

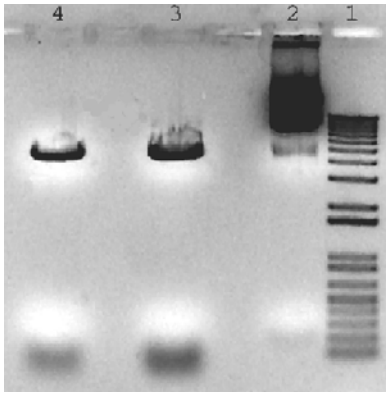


Fig 3.3 p+157SK+CGstem miniprep

1) *1Kb+ ladder.*
2) *Uncut plasmid isolated from transformant bacteria.*
3) *Plasmid isolated from transformant bacteria and cut with BspEI.*
4) *Plasmid isolated from transformant bacteria and cut with BspEI.*

3.1.1.2 Cloning CGstem into the chloroplast transformation vector

The plasmid obtained in the maxiprep from the first cloning stage (p+157SK+CGstem) was precipitated and cut with *Xho*I and *Xba*I. Two fragments were expected – one of about 3 kb and the other about 2kb). The 2kb fragment that contained the new insert (fig 2.2, materials and methods) was isolated by gel electrophoresis (fig 3.4) and its concentration was determined by dot spot analysis. In order to ensure isolation of the 2kb fragment alone (fig 3.4, lane 2), the 3kb fragment above it was cut out from the gel before the isolation of the 2kb fragment.

The plasmid pCrc32 was cut with *Xho*I and *Xba*I, resulting in two fragments of about 2kb and 9kb. The 9kb fragment was isolated by gel electrophoresis (fig 3.4) and its concentration was determined by dot spot analysis. The gel's resolution did not ensure that the 9kb fragment of pCrc32 did not contain any uncut of linearized (cut only with one enzyme) plasmid. This was therefore controlled in the same manner as the cutting of p+157SK+ (section 3.1.1.1) by checking transformation efficiencies of a ligation mix containing only the isolated 9kb band. Results showed little chance for religation without insert, proving most of pCrc32 was successfully double cut.

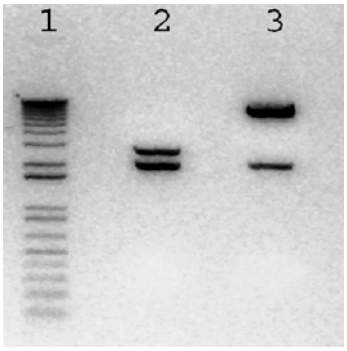


Fig 3.4 pCrc32 and p+157SK+CGstem cutting

1) *1Kb+ ladder.*
2) *p+157SK+CGstem cut with XhoI and XbaI.*
3) *pCrc32 was cut with XhoI and XbaI.*

Randomly picked colonies of cells transformed with the ligated 9kb and 2kb fragments were used for miniprep (fig 3.5).

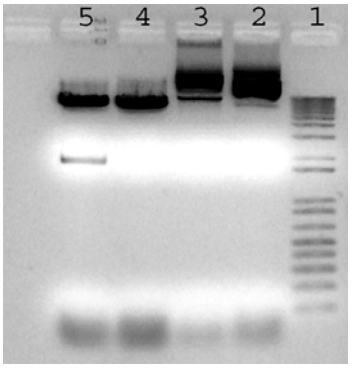


Fig 3.5 pCrc32CGstem miniprep

1) *1Kb+ ladder*
2) *The original pCrc32+157, uncut.*
3) *Plasmid obtained from transformant colony 1, uncut.*
4) *Plasmid from transformant colony 2, cut with XhoI. The linearized plasmid seems smaller than the large plasmid fragment in lane 5. This suggests that the obtained plasmid from this transformant colony has religated without incorporating the insert.*
5) *Plasmid from transformant colony 3, cut with XbaI and XhoI. The released insert verifies the plasmid had successfully incorporated the CGstem insert.*

No confirmation of correct insert orientation in the plasmid was needed, since the XhoI and XbaI leave distinctively different ends at their restriction sites and therefore allow only one insert orientation.

A maxiprep was done on transformant colony 3 from the miniprep, which tested positive for the CGstem insert, and was used for sequencing. The sequencing was successful (90% overall quality) and verified the incorporation of the insert into the plasmid.

3.1.2 CGstem analysis

Using the biolistics method, the transformation vector obtained from the maxiprep was used to transform the photosynthesis-deficient *Chlamydomonas* strain CC-373.
As mentioned before (section 1.2.1.1), the chloroplast of *C. reinhardtii* contains 50-80 genome copies. During transformation, insertion of DNA can occur in more than one of these copies. The selective pressure of exposure to light makes sure that surviving algae have incorporated the DNA complement to the cpDNA deletion found in this strain (fig 2.1, materials and methods), but due to the possibility of several homologous recombinations occurring, it does not guarantee the incorporation of the chimeric gene construct (*rbc*L5'UTR: GUS: *psa*B 3'region) that lies between the inverted repeat and the *atp*B gene in the transformation vector (fig 3.7).
In order to check for the presence and amount of the chimeric gene in transformants, the cultures' DNA was probed with a GUS probe using the slot blot method (fig 3.6). Three transformant cultures tested positive for GUS presence in their cpDNA. The signal from the GUS probe was relatively strong, suggesting the chimeric gene was incorporated into many copies of the chloroplast genome of these transformants.

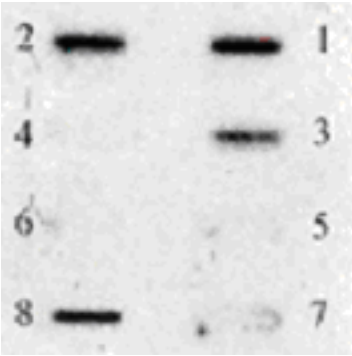


Fig 3.6 CGstem slot blot

1) *MU7 DNA hybridized to GUS probe (control).*
2) *Transformant algae 1 DNA hybridized to GUS probe.*
3) *Transformant algae 2 DNA hybridized to GUS probe.*
4) *Transformant algae 3 not containing GUS.*
5) *Transformant algae 4 not containing GUS.*
6) *Transformant algae 5 not containing GUS.*
7) *Transformant algae 6 not containing GUS.*
8) *Transformant algae 7 DNA hybridized to GUS probe.*

DNA from the cultures showing GUS was used in a southern blot, this time with a probe for the *atp*B gene (fig 3.8). The DNA was cut with the enzymes *Kpn*I and *Hind*III before it was run on the gel. Expected fragments containing *atp*B would be about 5kb and 3kb in size. The 5kb fragment represents chloroplast genome copies that have incorporated the chimeric gene construct, while the 3kb fragment represents chloroplast genome copies containing the deleted genes of CC-373, which are needed for photosynthesis, but not the chimeric gene tested (fig 3.7).

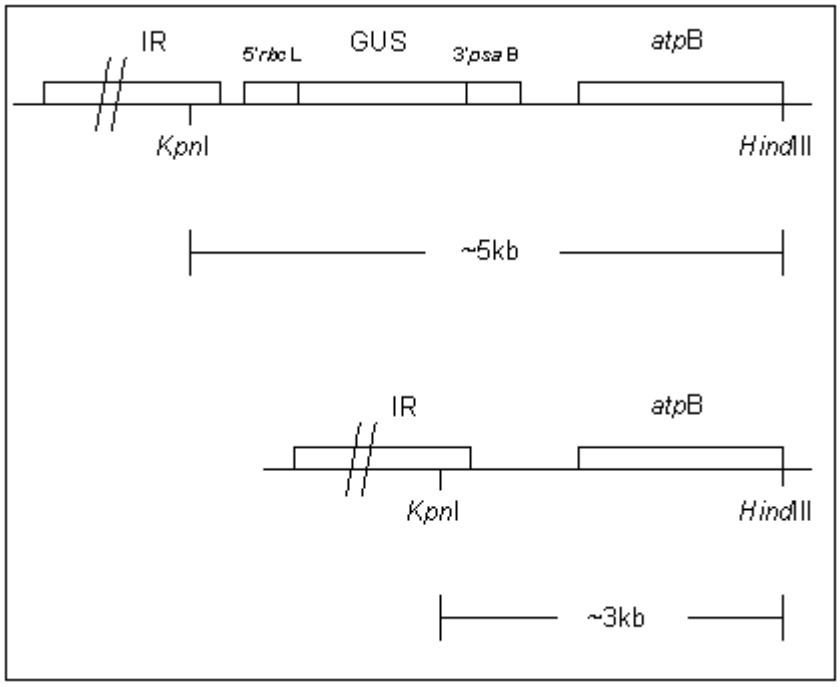


Fig 3.7 *The two possible atpB-containing fragments resulting from transformant DNA cutting with HindIII and KpnI.*

Of all three cultures tested, one culture showed only the 5kb fragment, which meant it was homoplasmic and all of its chloroplast genome copies contained the full chimeric gene construct (fig 3.8, lane 2).

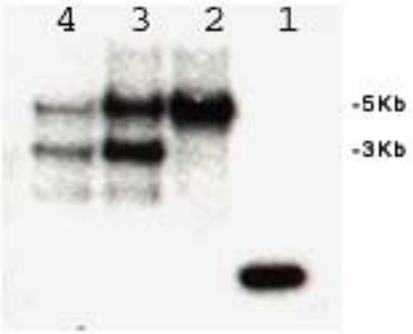


Fig 3.8 CGstem Southern blot

1) *1Kb+ ladder. The 1.6kb fragment of the ladder hybridizes with the atpB probe.*
2) *Transformant algae 1 DNA treated with KpnI and HindIII and hybridized to the atpB probe.*
3) *Transformant algae 2 DNA treated with KpnI and HindIII and hybridized to the atpB probe.*
4) *Transformant algae 7 DNA treated with KpnI and HindIII and hybridized to the atpB probe.*

After proving the presence of the chimeric gene in the transformants' DNA, the effect of the introduced change in the *rbc*L 5'UTR part of the chimeric gene could be tested. The homoplasmic transformant strain was the one tested in order to get the most accurate result. RNA was isolated as described in section 2.1.2.6. Chimeric gene transcript levels resulting from the chimeric transcript of transformant algae culture 1 were compared with those from the chimeric construct of MU7 transformant algae using a GUS (*uid*A transcript) probe.

Under both light and dark conditions, somewhat higher chimeric transcript levels were detected for transformant algae 1 in comparison to MU7 algae (fig 3.9).

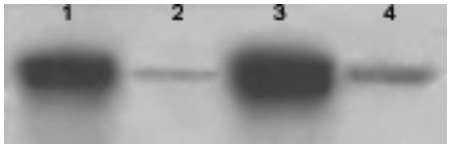


Fig 3.9 CGstem Northern blot

1) *MU7 uidA transcript level in the dark.*
2) *MU7 uidA transcript level in the light.*
3) *Transformant algae 1 uidA transcript level in the dark.*
4) *Transformant algae 1 uidA transcript level in the light.*

3.2 5'reverse construct analysis

The 5'reverse construct was cloned in a similar manner to the cloning procedure described for the CGstem construct, at the University of Valencia in Spain and analyzed for the second time in this work in order to verify previous results.

The transformation vector containing the construct was used to transform *C. reinhardtii* cells as described for the CGstem construct, resulting in light-tolerant (photosynthetic) transformants.

In a slot blot analysis, out of four cultures tested, one showed GUS presence in its DNA. The signal from the GUS probe was relatively strong suggesting high amounts of the chimeric gene incorporated into the chloroplast genome of this transformant strain (fig3.10).

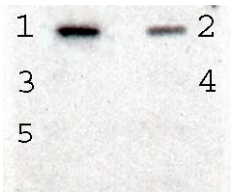


Fig3.10 5'reverse slot blot

1) *MU7 DNA hybridized with GUS.*
2) *Transformant 1 DNA hybridized with GUS.*
3) *Transformant 2 shows no GUS hybridization.*
4) *Transformant 3 shows no GUS hybridization.*
5) *Transformant 4 shows no GUS hybridization.*

DNA from the culture showing GUS was used in a Southern blot. The results showed only the 5kb fragment, proving homoplasmicity for the chimeric contruct (fig 3.11).

Fig 3.11 5'reverse Southern blot

1) *1Kb+ ladder. The 1.6kb fragment of the ladder hybridizes with atpB probe.*
2) *Transformant algae 1 cpDNA treated with KpnI and HindIII and hybridized to the atpB probe*.

A northern blot done on RNA isolated from transformant algae 1 (fig 3.12) showed similar, though slightly higher, *uid*A transcript levels in comparison to those found in MU7, which contained the unchanged *rbc*L 5'UTR, under both light and dark growth.

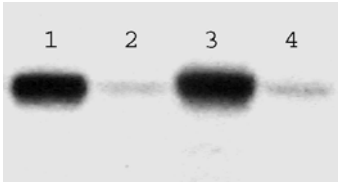


Fig 3.12 5'reverse Northern blot

1) *MU7 uidA transcript level in the dark.*
2) *MU7 uidA transcript level in the light.*
3) *Transformant algae 1 uidA transcript level in the dark.*
4) *Transformant algae 1 uidA transcript level in the light.*

4. DISCUSSION

The constructs tested in this work contain changes in the 5'UTR of the *Chlamydomonas rbc*L gene. 5'UTRs are known to contain determinants important for transcript stability and translatability (Drager et al. 1999; Higgs et al. 1999; Nickelsen et al. 1999; Anthonisen et al. 2001; Salvador et al. 2004).

The *C. reinhardtii rbc*L 5'UTR contains a stabilizing sequence element made of nucleotides +38 to +47 (+1 being transcription start point) (Anthonisen et al. 2001). This element lies within a predicted secondary structure consisting of a large stem-loop followed by a smaller stem-loop downstream of it (Anthonisen et al. 2001) (fig 4.1).

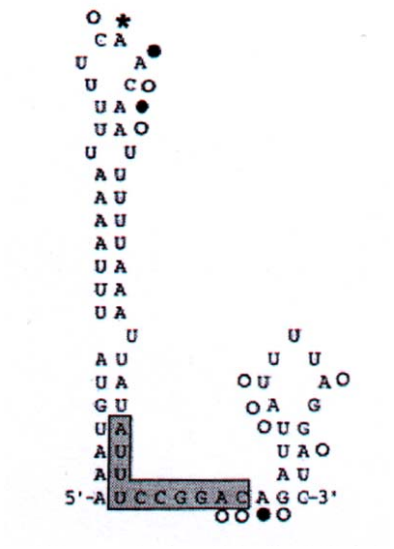


Fig 4.1 The predicted structure of nucleotides +1 to +63 of the C. reihardtii rbcL 5'UTR *(Anthonisen et al. 2001). Stability element (+38 to +47) marked.*

It has been previously observed that chimeric transcripts containing only the 5'UTR part of the *C.reinhardtii rbc*L gene are substantially less stable in light than they are in the dark. It was also established that the first 252 nucleotides of the *C. reinhardtii rbc*L-coding region stabilize the transcript in the light (Salvador et al. 1993). A conserved element that stabilizes the transcript in the light was identified later on between

nucleotides +329 to +334 (fig 4.2) (Singh et al. 2001). Mutations in the preserved +38 to +47 stability element of the *rbc*L 5'UTR destabilize the chimeric transcript. However, in chimeric transcripts that do not contain the *rbc*L coding region, which includes the +329 to +334 element, these mutations in the 5'UTR stability element do not affect the difference seen in transcript stability under dark and light conditions (Anthonisen et al. 2001). Therefore, two chloroplast RNA degradation pathways exist, one light-dependent (photo-accelerated) degradation, from which the +329 to +334 element protects, and the other light-independent, in which the +38 and +47 element plays a role.

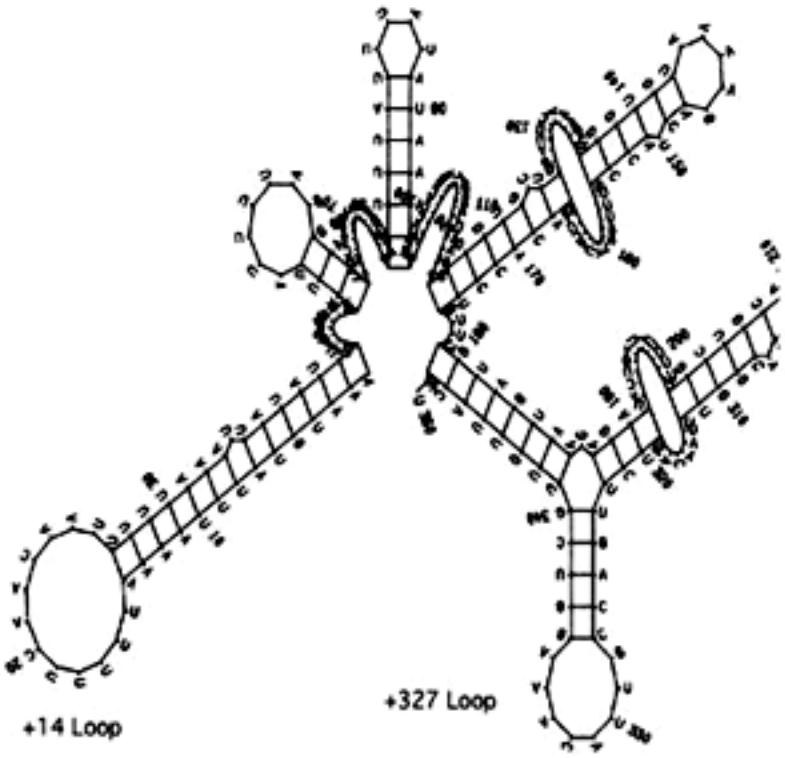


Fig 4.2 *Prediction of the structure of nucleotides +1 to +350 of the C.reinhardtii rbcL gene. The +327 loop contains the +329 to +334 stability element. (Singh et al. 2001)*

In *C. reinhardtii rbc*L, in vivo studies revealed a compensation mechanism, balancing increased transcription rate in the light with lower transcript stability, and decreased transcription rate in the dark with higher transcript stability, the overall result being a

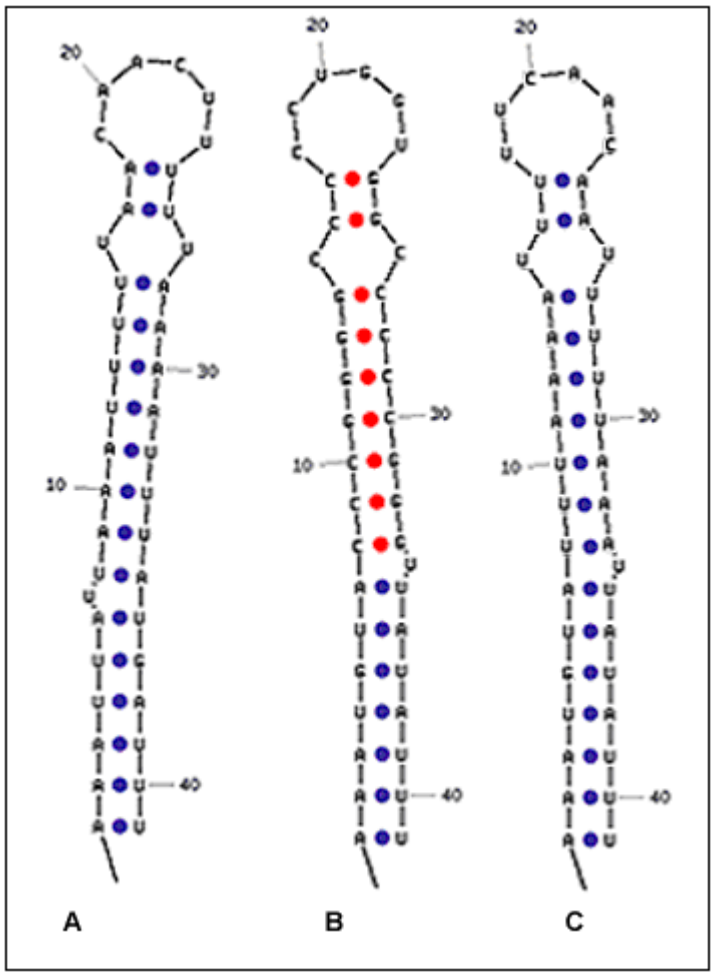
steady state in transcript abundance (Salvador et al. 1993). This pattern of chloroplast transcript stability and transcription rate that differ in dark and light conditions is also found in the chloroplasts of higher model organisms (Shiina et al. 1998). It is likely that the environmental signal of light is carried to the RNA degradation mechanism by redox carriers, since studies in *C. reinhardtii* show photosynthetic non-cyclic electron transport to be crucial for light-dependent RNA degradation (Salvador et al. 1999).

The constructs tested in this work were planned so that the exact structure of the stem-loop is maintained, while its entire sequence is changed (section 1.5) (fig 4.3). Both constructs did not cause a change in the dark/light stability ratio of the chimeric gene transcript. They did, however, show slightly higher stability than the chimeric gene transcript of the MU7 transformant, which contains the unchanged *rbc*L 5'UTR. This is the result of a transcription enhancer that is present in the tested transformation constructs but not in the MU7 construct.

The CGstem construct has an increased number of hydrogen bonds in comparison to the original stem-loop sequence. This could potentially give a biased result when it comes to transcript stability by providing a more stable stem-loop, but since the 5'reverse construct with its unchanged number of hydrogen bonds in the stem-loop gives similar results, it can be concluded that this is not a major factor in the chimeric transcript's stability.

It is established that mutations in this region of the stem-loop do not affect the transcription rate (Salvador et al. 2004), which implies that the results of this work reflect changes in transcript stability alone.

It can therefore be concluded that the nucleotide sequence at position +5 to +37 of large stem-loop in the *C.reinhardtii rbc*L does not contain significant elements for transcript stability. Nucleotides +38 to +41 and +1 to +4 were not changed in the tested constructs in order to preserve the sequence and structure of the +38 to +47 stability element. These results do not allow conclusion regarding the predicted role of the stem-loop structure in transcript stability, but other constructs testing this region of the *rbc*L 5'UTR confirm the importance of this structure (Salvador et al. 2004).



D

rbcL 5'UTR (+1 to +46)
5'AAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTTCCGGA3'
5'reverse (+1 to +46)
5'AAATTATTAAATTTTTTAACAACTTTTTAAAATTTATGATTTCCGGA3'
CGstem (+1 to +46)
5'AAATGCGCCCGGGGCCCCCTGGTGGCCCCCGGGCCGTATTTCCGGA3'

Fig 4.3 The changes made in the C. reinhardtii rbcL 5'UTR
A and B *Showing the structural change made in the large 5'UTR stem-loop in the 5'reverse and CGstem constructs, respectively, as predicted by mfold (Zuker et al. 1999).*
C *The structure of the original large stem-loop of the rbcL 5'UTR, as predicted by mfold (Zuker et al. 1999).*
D *The sequences (nucleotides +1 to +46) of the original rbcL 5'UTR and the two constructs.*

The large stem-loop of the *C. reinhardtii rbc*L 5'UTR has been previously claimed to participate in light-dependent degradation through a nuclease target in nucleotides +27 to +41 and possibly also through an indirect interaction of the nucleotides at the top of the stem-loop with the coding-region stem-loop containing the +329 to +334 sequence element, preventing access to the postulated nuclease target (Singh et al. 2001). The results of this work do not support these suggestions. The nucleotides at the top of the large stem-loop in the 5'UTR are not conserved and are therefore not likely to serve as a target for a protein that could interact with other RNA-binding proteins and change the tertiary structure of the molecule. The possibility of the +27 to +41 nucleotides serving as a nuclease target or a target for indirect interaction with another stem-loop structure is unlikely since the sequence of nucleotides +5 to +37 is insignificant for transcript stability, and since nucleotides +38 to +41 belong to a stabilizing element and mutations in them destabilize transcripts, rather than stabilize them. However, the fact that the 5'UTR stem-loop structure is of importance for transcript stability (Salvador et al. 2004) and the location of the +38 to +41 of the stability sequence element as part of the stem-loop, may imply that some significant indirect interactions with other regions in the *C.reinhardtii rbc*L transcript do occur via RNA-binding proteins. This seems also likely in light of the fact that the endogenous *rbc*L transcripts have half-lives that are substantially longer than those of the chimeric reporter genes studied, even when the first 252 nucleotides of the gene's coding region are included in the constructs (Salvador et al. 1993; Nickelsen et al. 1994).

Although several crucial elements for the regulation of the *rbc*L are well defined, it is likely that other elements active in the regulation of this gene's function still remain unknown. In addition to the study of gene structure, the thorough study of the sequence and structure of this gene's and other chloroplast genes' transcripts, provides an opportunity to gain insight in the evolutionary development of protein and RNA interactions as well as the development of organelle communication.

Agrawal, G. K., Kato, H., Asayama, M., and Shirai, M. (2001). An AU-box motif upstream of the SD sequence of light-dependent psbA transcripts confers mRNA instability in darkness in cyanobacteria. *Nucleic Acids Res* **29**, 1835-43.

Anthonisen, I. L., Salvador, M. L., and Klein, U. (2001). Specific sequence elements in the 5' untranslated regions of rbcL and atpB gene mRNas stabilize transcripts in the chloroplast of Chlamydomonas reinhardtii. *Rna* **7**, 1024-33.

Barkan, A. (1989). Tissue-dependent plastid RNA splicing in maize: transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. *Plant Cell* **1**, 437-45.

Barkan, A. (1993). Nuclear Mutants of Maize with Defects in Chloroplast Polysome Assembly Have Altered Chloroplast RNA Metabolism. *Plant Cell* **5**, 389-402.

Barkan, A., Walker, M., Nolasco, M., and Johnson, D. (1994). A nuclear mutation in maize blocks the processing and translation of several chloroplast mRNAs and provides evidence for the differential translation of alternative mRNA forms. *Embo J* **13**, 3170-81.

Bendich, A. J. (1987). Why do chloroplasts and mitochondria contain so many copies of their genome? *Bioessays* **6**, 279-82.

Birky, C. W., Jr. (1983). Relaxed cellular controls and organelle heredity. *Science* **222**, 468-75.

Birky, C. W., Jr. (1995). Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc Natl Acad Sci U S A* **92**, 11331-8.

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**, 123-32.

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**, 339-49

Bock, R. (2000). Sense from nonsense: how the genetic information of chloroplasts is altered by RNA editing. *Biochimie* **82**, 549-57.

Bock, R., Hagemann, R., Kossel, H., and Kudla, J. (1993). Tissue- and stage-specific modulation of RNA editing of the psbF and psbL transcript from spinach plastids--a new regulatory mechanism? *Mol Gen Genet* **240**, 238-44.

Bock, R., Kossel, H., and Maliga, P. (1994). Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype. *Embo J* **13**, 4623-8.

Bollenbach, T. J., Schuster, G., and Stern, D. B. (2004). Cooperation of endo- and exoribonucleases in chloroplast mRNA turnover. *Prog Nucleic Acid Res Mol Biol* **78**, 305-37.

Boudreau, E., Otis, C., and Turmel, M. (1994). Conserved gene clusters in the highly rearranged chloroplast genomes of Chlamydomonas moewusii and Chlamydomonas reinhardtii. *Plant Mol Biol* **24**, 585-602.

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**, 1534-8.

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**, 1145-53.

Calie, P. J., and Manhart, J. R. (1994). Extensive sequence divergence in the 3' inverted repeat of the chloroplast rbcL gene in non-flowering land plants and algae. *Gene* **146**, 251-6.

Danon, A., and Mayfield, S. P. (1994). ADP-dependent phosphorylation regulates RNA-binding in vitro: implications in light-modulated translation. *Embo J* **13**, 2227-35.

Dellaporta, S.L., Wood, J., Hicks, J.B. (1983). A plant DNA minipreparation: Version II. *Plant Mol Biol Rep* **1**, 19-21.

Dent, R. M., Han, M., and Niyogi, K. K. (2001). Functional genomics of plant photosynthesis in the fast lane using Chlamydomonas reinhardtii. *Trends Plant Sci* **6**, 364-71.

Deshpande, N. N., Bao, Y., and Herrin, D. L. (1997). Evidence for light/redox-regulated splicing of psbA pre-RNAs in Chlamydomonas chloroplasts. *Rna* **3**, 37-48.

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.

Drager, R. G., Higgs, D. C., Kindle, K. L., and Stern, D. B. (1999). 5' to 3' exoribonucleolytic activity is a normal component of chloroplast mRNA decay pathways. *Plant J* **19**, 521-31.

Drager, R. G., Zeidler, M., Simpson, C. L., and Stern, D. B. (1996). A chloroplast transcript lacking the 3' inverted repeat is degraded by 3'-->5' exoribonuclease activity. *Rna* **2**, 652-63.

Drapier, D., Girard-Bascou, J., Stern, D. B., and Wollman, F. A. (2002). A dominant nuclear mutation in Chlamydomonas identifies a factor controlling chloroplast mRNA stability by acting on the coding region of the atpA transcript. *Plant J* **31**, 687-97.

Eibl, C., Zou, Z., Beck, a., Kim, M., Mullet, J., and Koop, H. U. (1999). In vivo analysis of plastid psbA, rbcL and rpl32 UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. *Plant J* **19**, 333-45.

Esposito, D., Higgs, D. C., Drager, R. G., Stern, D. B., and Girard-Bascou, J. (2001). A nucleus-encoded suppressor defines a new factor which can promote petD mRNA stability in the chloroplast of Chlamydomonas reinhardtii. *Curr Genet* **39**, 40-8.

Frey, J. E. (1999). Genetic flexibility of plant chloroplasts. *Nature* **398**, 115-6.

Freyer, R., Hoch, B., Neckermann, K., Maier, R. M., and Kossel, H. (1993). RNA editing in maize chloroplasts is a processing step independent of splicing and cleavage to monocistronic mRNAs. *Plant J* **4**, 621-9.

Gillham, N. W., Boynton, J. E., and Hauser, C. R. (1994). Translational regulation of gene expression in chloroplasts and mitochondria. *Annu Rev Genet* **28**, 71-93.

Grossman, A. R. (2000). Chlamydomonas reinhardtii and photosynthesis: genetics to genomics. *Curr Opin Plant Biol* **3**, 132-7.

Harris, E. H. (1989). The Chlamydomonas sourcebook: A comprehensive guide to biology and laboratory use. *Academic Press. San Diego. California.*

Harris, E. H. (2001). Chlamydomonas as a Model Organism. *Annu Rev Plant Physiol Plant Mol Biol* **52**, 363-406.

Hayes, R., Kudla, J., and Gruissem, W. (1999). Degrading chloroplast mRNA: the role of polyadenylation. *Trends Biochem Sci* **24**, 199-202.

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**, 1132-41.

Hess, W. R., Hoch, B., Zeltz, P., Hubschmann, T., Kossel, H., and Borner, T. (1994). Inefficient rpl2 splicing in barley mutants with ribosome-deficient plastids. *Plant Cell* **6**, 1455-65.

Higgs, D.C., Shapiro, R.S., Kindle, K.L. and Stern, D.B. (1999). Small cis-acting sequences that specify secondary structures in a chloroplast mRNA are essential for RNA stability and translation. *Mol Cell Biol* **19**, 8479-91.

Hirose, T., and Sugiura, M. (1997). Both RNA editing and RNA cleavage are required for translation of tobacco chloroplast ndhD mRNA: a possible regulatory mechanism for the expression of a chloroplast operon consisting of functionally unrelated genes. *Embo J* **16**, 6804-11.

Hirose, T., and Sugiura, M. (2001). Involvement of a site-specific trans-acting factor and a common RNA-binding protein in the editing of chloroplast mRNAs: development of a chloroplast in vitro RNA editing system. *Embo J* **20**, 1144-52.

Hoch, B., Maier, R. M., Appel, K., Igloi, G. L., and Kossel, H. (1991). Editing of a chloroplast mRNA by creation of an initiation codon. *Nature* **353**, 178-80.

Holloway, S. P., and Herrin, D. L. (1998). Processing of a composite large subunit rRNA. Studies with chlamydomonas mutants deficient in maturation of the 23s-like rrna. *Plant Cell* **10**, 1193-206.

Horlitz, M., and Klaff, P. (2000). Gene-specific trans-regulatory functions of magnesium for chloroplast mRNA stability in higher plants. *J Biol Chem* **275**, 35638-45.

Hubschmann, T., Hess, W. R., and Borner, T. (1996). Impaired splicing of the rps12 transcript in ribosome-deficient plastids. *Plant Mol Biol* **30**, 109-23.

Jenkins, B. D., Kulhanek, D. J., and Barkan, A. (1997). Nuclear mutations that block group II RNA splicing in maize chloroplasts reveal several intron classes with distinct requirements for splicing factors. *Plant Cell* **9**, 283-96.

Joyce, P. B., and Gray, M. W. (1989). Chloroplast-like transfer RNA genes expressed in wheat mitochondria. *Nucleic Acids Res* **17**, 5461-76.

Karcher, D., and Bock, R. (1998). Site-selective inhibition of plastid RNA editing by heat shock and antibiotics: a role for plastid translation in RNA editing. *Nucleic Acids Res* **26**, 1185-90.

Karcher, D., and Bock, R. (2002). Temperature sensitivity of RNA editing and intron splicing reactions in the plastid ndhB transcript. *Curr Genet* **41**, 48-52.

Kim, J., and Mayfield, S. P. (1997). Protein disulfide isomerase as a regulator of chloroplast translational activation. *Science* **278**, 1954-7.

Kim, M., Christopher, D. A., and Mullet, J. E. (1993). Direct evidence for selective modulation of psbA, rpoA, rbcL and 16S RNA stability during barley chloroplast development. *Plant Mol Biol* **22**, 447-63.

Klaff, P. (1995). mRNA decay in spinach chloroplasts: psbA mRNA degradation is initiated by endonucleolytic cleavages within the coding region. *Nucleic Acids Res* **23**, 4885-92.

Klaff, P., and Gruissem, W. (1991). Changes in Chloroplast mRNA Stability during Leaf Development. *Plant Cell* **3**, 517-529.

Klein, T. M., Wolf, E. D., Wu, R., Sanford, J.C. (1987). High-velocity microprojectiles+ for delivering nucleic acids into living cells. *Nature* **327**, 70 – 73.

Kudla, J., and Bock, R. (1999). RNA editing in an untranslated region of the Ginkgo chloroplast genome. *Gene* **234**, 81-6.

Kulkarni, R. D., and Golden, S. S. (1997). mRNA stability is regulated by a coding-region element and the unique 5' untranslated leader sequences of the three Synechococcus psbA transcripts. *Mol Microbiol* **24**, 1131-42.

Lambowitz, A. M., and Perlman, P. S. (1990). Involvement of aminoacyl-tRNA synthetases and other proteins in group I and group II intron splicing. *Trends Biochem Sci* **15**, 440-4.

Lee, H., Bingham, S. E., and Webber, A. N. (1996). Function of 3' non-coding sequences and stop codon usage in expression of the chloroplast psaB gene in Chlamydomonas reinhardtii. *Plant Mol Biol* **31**, 337-54.

Lee, J., and Herrin, D. L. (2003). Mutagenesis of a light-regulated psbA intron reveals the importance of efficient splicing for photosynthetic growth. *Nucleic Acids Res* **31**, 4361-72.

Lefebvre, P. A., and Silflow, C. D. (1999). Chlamydomonas: the cell and its genomes. *Genetics* **151**, 9-14.

Leister, D. (2003). Chloroplast research in the genomic age. *Trends Genet* **19**, 47-56.

Levy, H., Kindle, K. L., and Stern, D. B. (1999). Target and specificity of a nuclear gene product that participates in mRNA 3'-end formation in Chlamydomonas chloroplasts. *J Biol Chem* **274**, 35955-62.

Liere, K., and Link, G. (1997). Chloroplast endoribonuclease p54 involved in RNA 3'-end processing is regulated by phosphorylation and redox state. *Nucleic Acids Res* **25**, 2403-8.

Lilly, J. W., Maul, J. E., and Stern, D. B. (2002). The Chlamydomonas reinhardtii organellar genomes respond transcriptionally and post-transcriptionally to abiotic stimuli. *Plant Cell* **14**, 2681-706.

Lisitsky, I., and Schuster, G. (1995). Phosphorylation of a chloroplast RNA-binding protein changes its affinity to RNA. *Nucleic Acids Res* **23**, 2506-11.

Maier, R. M., Hoch, B., Zeltz, P., and Kossel, H. (1992). Internal editing of the maize chloroplast ndhA transcript restores codons for conserved amino acids. *Plant Cell* **4**, 609-16.

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**, 614-28.

Maul, J. E., Lilly, J. W., Cui, L., dePamphilis, C. W., Miller, W., Harris, E. H., and Stern, D. B. (2002). The Chlamydomonas reinhardtii plastid chromosome: islands of genes in a sea of repeats. *Plant Cell* **14**, 2659-79.

Mayfield, S. P. (1990). Chloroplast gene regulation: interaction of the nuclear and chloroplast genomes in the expression of photosynthetic proteins. *Curr Opin Cell Biol* **2**, 509-13.

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

McCormac, D. J., Litz, H., Wang, J., Gollnick, P. D., and Berry, J. O. (2001). Light-associated and processing-dependent protein binding to 5' regions of rbcL mRNA in the chloroplasts of a C4 plant. *J Biol Chem* **276**, 3476-83.

Memon, A. R., Meng, B., and Mullet, J. E. (1996). RNA-binding proteins of 37/38 kDa bind specifically to the barley chloroplast psbA 3'-end untranslated RNA. *Plant Mol Biol* **30**, 1195-205.

Menand, B., Marechal-Drouard, L., Sakamoto, W., Dietrich, A., and Wintz, H. (1998). A single gene of chloroplast origin codes for mitochondrial and chloroplastic methionyl-tRNA synthetase in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **95**, 11014-9.

Merchant, S., and Bogorad, L. (1986). Regulation by copper of the expression of plastocyanin and cytochrome c552 in Chlamydomonas reinhardi. *Mol Cell Biol* **6**, 462-9.

Miyamoto, T., Obokata, J., and Sugiura, M. (2002). Recognition of RNA editing sites is directed by unique proteins in chloroplasts: biochemical identification of cis-acting elements and trans-acting factors involved in RNA editing in tobacco and pea chloroplasts. *Mol Cell Biol* **22**, 6726-34.

Monde, R. A., Schuster, G., and Stern, D. B. (2000). Processing and degradation of chloroplast mRNA. *Biochimie* **82**, 573-82.

Nakamura, T., Ohta, M., Sugiura, M., and Sugita, M. (1999). Chloroplast ribonucleoproteins are associated with both mRNAs and intron-containing precursor tRNAs. *FEBS Lett* **460**, 437-41.

Nakamura, T., Ohta, M., Sugiura, M., and Sugita, M. (2001). Chloroplast ribonucleoproteins function as a stabilizing factor of ribosome-free mRNAs in the stroma. *J Biol Chem* **276**, 147-52.

Nickelsen, J., Fleischmann, M., Boudreau, E., Rahire, M., and Rochaix, J. D. (1999). Identification of cis-acting RNA leader elements required for chloroplast psbD gene expression in Chlamydomonas. *Plant Cell* **11**, 957-70.

Nickelsen, J., and Kuck, U. (2000). The unicellular green alga Chlamydomonas reinhardtii as an experimental system to study chloroplast RNA metabolism. *Naturwissenschaften* **87**, 97-107.

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**, 3182-91.

Peeters, N. M., and Hanson, M. R. (2002). Transcript abundance supercedes editing efficiency as a factor in developmental variation of chloroplast gene expression. *Rna* **8**, 497-511.

Race, H. L., Herrmann, R. G., and Martin, W. (1999). Why have organelles retained genomes? *Trends Genet* **15**, 364-70.

Reinbothe, S., Reinbothe, C., Heintzen, C., Seidenbecher, C., and Parthier, B. (1993). A methyl jasmonate-induced shift in the length of the 5' untranslated region impairs translation of the plastid rbcL transcript in barley. *Embo J* **12**, 1505-12.

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

Rochaix, J. D. (1995). Chlamydomonas reinhardtii as the photosynthetic yeast. *Annu Rev Genet* **29**, 209-30.

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**, 676-83.

Rott, R., Liveanu, V., Drager, R. G., Higgs, D., Stern, D. B., and Schuster, G. (1999). Altering the 3 UTR endonucleolytic cleavage site of a Chlamydomonas chloroplast mRNA affects 3-end maturation in vitro but not in vivo. *Plant Mol Biol* **40**, 679-86.

Rott, R., Liveanu, V., Drager, R. G., Stern, D. B., and Schuster, G. (1998). The sequence and structure of the 3'-untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability. *Plant Mol Biol* **36**, 307-14.

Ruf, S., and Kossel, H. (1997). Tissue-specific and differential editing of the two ycf3 editing sites in maize plastids. *Curr Genet* **32**, 19-23.

Ruf, S., Zeltz, P., and Kossel, H. (1994). Complete RNA editing of unspliced and dicistronic transcripts of the intron-containing reading frame IRF170 from maize chloroplasts. *Proc Natl Acad Sci U S A* **91**, 2295-9.

Saldanha, R., Mohr, G., Belfort, M., and Lambowitz, A. M. (1993). Group I and group II introns. *Faseb J* **7**, 15-24.

Salvador, M. L., and Klein, U. (1999). The redox state regulates RNA degradation in the chloroplast of Chlamydomonas reinhardtii. *Plant Physiol* **121**, 1367-74.

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 U S A* **90**, 1556-60.

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**, 213-9.

Sambrook, J. and Russell, D.W. (2001). Molecular cloning: A laboratory manual. *Cold Spring Harbor Laboratory Press 3rd ed. New York*.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463-7.

Sasaki, Y., Kozaki, A., Ohmori, A., Iguchi, H., and Nagano, Y. (2001). Chloroplast RNA editing required for functional acetyl-CoA carboxylase in plants. *J Biol Chem* **276**, 3937-40.

Schuster, G., Lisitsky, I., and Klaff, P. (1999). Polyadenylation and degradation of mRNA in the chloroplast. *Plant Physiol* **120**, 937-44.

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**, 1001-11.

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**, 1713-22.

Singh, M., Boutanaev, A., Zucchi, P., and Bogorad, L. (2001). Gene elements that affect the longevity of rbcL sequence-containing transcripts in Chlamydomonas reinhardtii chloroplasts. *Proc Natl Acad Sci U S A* **98**, 2289-94.

Smith, A.C., Purton, S. (2002). The transcriptional apparatus of algal plastids. *J Phyc* **37**, 301-311.

Smith, H. C., Gott, J. M., and Hanson, M. R. (1997). A guide to RNA editing. *Rna* **3**, 1105-23.

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**, 1145-57.

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**, 18742-50.

Stern, D. B., and Kindle, K. L. (1993). 3'end maturation of the Chlamydomonas reinhardtii chloroplast atpB mRNA is a two-step process. *Mol Cell Biol* **13**, 2277-85.

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**, 285-97.

Sueoka, N. (1960). Mitotic replication of deoxyribonucleic acid in Chlamydomonas reinhardtii. *Proc Natl Acad Sci U S A* **46**, 83-91.

Turmel, M., Otis, C., and Lemieux, C. (1999). The complete chloroplast DNA sequence of the green alga Nephroselmis olivacea: insights into the architecture of ancestral chloroplast genomes. *Proc Natl Acad Sci U S A* **96**, 10248-53.

Vaistij, F. E., Boudreau, E., Lemaire, S. D., Goldschmidt-Clermont, M., and Rochaix, J. D. (2000). Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast psbB/psbT/psbH gene cluster in Chlamydomonas reinhardtii. *Proc Natl Acad Sci U S A* **97**, 14813-8.

Yohn, C. B., Cohen, A., Danon, A., and Mayfield, S. P. (1996). Altered mRNA binding activity and decreased translational initiation in a nuclear mutant lacking translation of the chloroplast psbA mRNA. *Mol Cell Biol* **16**, 3560-6.

Yoshinaga, K., Iinuma, H., Masuzawa, T., and Uedal, K. (1996). Extensive RNA editing of U to C in addition to C to U substitution in the rbcL transcripts of hornwort chloroplasts and the origin of RNA editing in green plants. *Nucleic Acids Res* **24**, 1008-14.

Yu, W., and Spreitzer, R. J. (1992). Chloroplast heteroplasmicity is stabilized by an amber-suppressor tryptophan tRNA(CUA). *Proc Natl Acad Sci U S A* **89**, 3904-7.

Zeltz, P., Hess, W. R., Neckermann, K., Borner, T., and Kossel, H. (1993). Editing of the chloroplast rpoB transcript is independent of chloroplast translation and shows different patterns in barley and maize. *Embo J* **12**, 4291-6.

Zerges, W., Auchincloss, A. H., and Rochaix, J. D. (2003). Multiple translational control sequences in the 5' leader of the chloroplast psbC mRNA interact with nuclear gene products in Chlamydomonas reinhardtii. *Genetics* **163**, 895-904.

Zito, F., Kuras, R., Choquet, Y., Kossel, H., and Wollman, F. A. (1997). Mutations of cytochrome b6 in Chlamydomonas reinhardtii disclose the functional significance for a proline to leucine conversion by petB editing in maize and tobacco. *Plant Mol Biol* **33**, 79-86.

Zuker, M., Mathewes, D.H., Turner, D.H. (1999). Algorithms and thermodynamics for RNA secondary structure prediction; A practical guide. In Barciszewski, J., Clarck, BCF (eds). *RNA biochemistry and biotechnology. NATO ASI Series, Kluwer Academic Publishers*, pp 11-43.