Thesis for the Master's degree in Molecular Biosciences
Main field of study in Molecular Biology

60 study points

Destabilitation of *Chlamydomonas reinhardtii* *rbcL* transcripts by changes in their 5'UTR

Marit Nedkvitne Leinaas

Department of Molecular Biosciences
Faculty of mathematics and natural sciences

UNIVERSITY OF OSLO

12/2008

Acknowledgements

The master project was performed at the department of Molecular Biology at the university of Oslo in Professor Uwe Klein`s laboratory.

I would like to thank my supervisor Professor Uwe Klein for all his help in the laboratory and his patient guidance when I was writing this thesis. He has always taken the time assist to me in the laboratory and to answer my questions.

I would also like to thank Jahn Frederik Kjellin and Matilde Margrete Leinaas Kjellin for their support.

Oslo, December 2008

Marit Nedkvitne Leinaas

SUMMARY:

This study is part of a project where the *Chlamydomonas reindhardtii rbcL* 5'UTR is analyzed for its effect on transcript stability. Two different mutated *rbcL* 5'UTRs were fused to GUS reporter genes, and introduced into the *Chlamydomonas* chloroplast genome. *In vivo* transcript accumulation was determined by northern blot assay.

One of the 5'UTR modifications was an addition of 9 nucleotides to the 5'terminus of the transcript and a deletion of the nucleotide at the original start of transcription. The other mutation changed the nucleotide sequence in a 5'UTR stem-loop without changing its secondary structure. It was found that both modifications rendered the transcripts unstable, and significantly reduced accumulation of GUS transcripts.

Table of Contents

1. Introduction

1.1 The Chloroplast

1.1.1 The chloroplast

Chloroplasts are organelles found in plant cells and eukaryotic algae. The chloroplast belongs to the organelle group called plastids. Chloroplasts are usually 2 to 10 μm in diameter, and most chloroplasts have a double membrane. The matrix inside the chloroplast is called the stroma. Within the stroma lie plastid DNA, ribosomes, and stacks of thylakoids. A thylakoid stack is called a granum (Figure 1-1). Photosynthesis takes place in the thylakoid membrane.

Plastids are believed to originate from an endosymbiosis with cyanobacteria that allowed eukaryotes to carry out oxygenic photosynthesis (Cavalier-Smith, 2000). The mitochondrion is also believed to originate from a similar endosymbiotic event. The genome of organelles is considerably reduced compared to that of their evolutionary ancestors (Hoffmeister & Martin, 2003; Martin et al., 2002). Most genes in organelle genomes have been lost or transferred to the nucleus. In plants and algae this gene-transfer is believed to be an ongoing process. There have also been found examples of mitochondrion-plastid gene transfer, and transfer of genes from the nucleus to organelles. The influence from the nucleus has resulted in a eukaryote-like plastid genome organization (e.g., intron invasion and the presence of maturases) and regulatory units to genes (Maul et al., 2002). Most genes needed for mitochondrial and plastid functions are located in the nucleus (~95%). Chloroplasts typically contain only 100-200 genes, whereas cyanobacteria often contain more than 1500 genes. Chloroplast proteins encoded in the nucleus are transported to the chloroplast by a unique translocation system, which is inherited, in part, from the endosymbiotic ancestor (Soll & Schleiff, 2004).

The chloroplast is a semi-autonomous organelle that contains the biochemical machinery necessary to replicate and transcribe its own genome and to synthesize protein. Most of the genes retained in the chloroplast are those encoding components

of the photosynthetic apparatus and the gene expression apparatus of the plastid (Barbrook et al, 2006). One of the reasons some genes are not relocated to the nucleus might be that synthesis of these proteins in the chloroplast makes redox-regulated expression a direct way to rapidly regulate these genes by redox signals from the electron transport chain (Bock & Timmis, 2008; Race et al, 1999). Another reason might be that the plastid location allows those genes to be regulated as part of global plastid gene-regulation e.g. during plastid development.

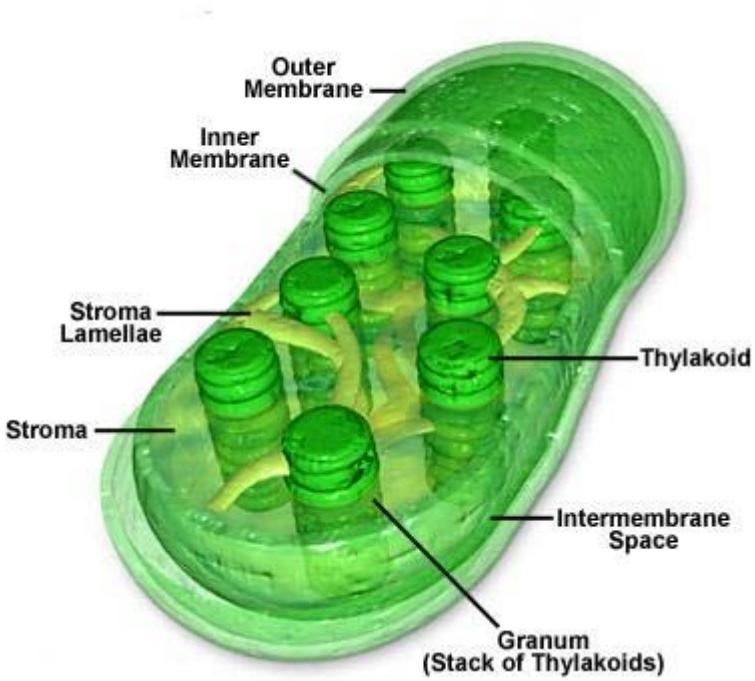


Figure 1-1: Plant cell chloroplast structure. *Photosynthesis takes place in the thylakoid membrane. Illustration from http://micro.magnet.fsu.edu.*

1.1.2 Photosynthesis

Located in the thylakoid membrane are pigments (in the antenna complex) that capture and transfer photons to a reaction centre, where they ionize chlorophyll molecules. This produces excited electrons, which are used in an electron flow chain to convert ADP to ATP and to reduce NADP+ to NADPH while producing oxygen gas from water. These reactions are known as the light reactions of photosynthesis

(Figure 1-2). In the algal chloroplast ATP is generated via the transmembrane enzyme CF_0CF_1 ATPase. The Calvin cycle (dark reactions) fixes carbon from CO_2 into organic compounds. The enzymes in the cycle are not membrane-bound but soluble in the stroma. One of the enzymes is RuBisCO (Ribulose-1.5-bisphosphate carboxylase / oxygenase), which catalyzes the first major step of carbon fixation. RuBisCO is thought to be the most abundant protein in the world, and usually consists of two subunits, called the large chain and the small chain. The *rbcL* gene encodes the large chain. The reducing power and ATP produced by the light reactions have through several studies been linked to regulation of mRNA degradation and other parts of the molecular machinery in chloroplasts (Salvador & Klein, 1999).

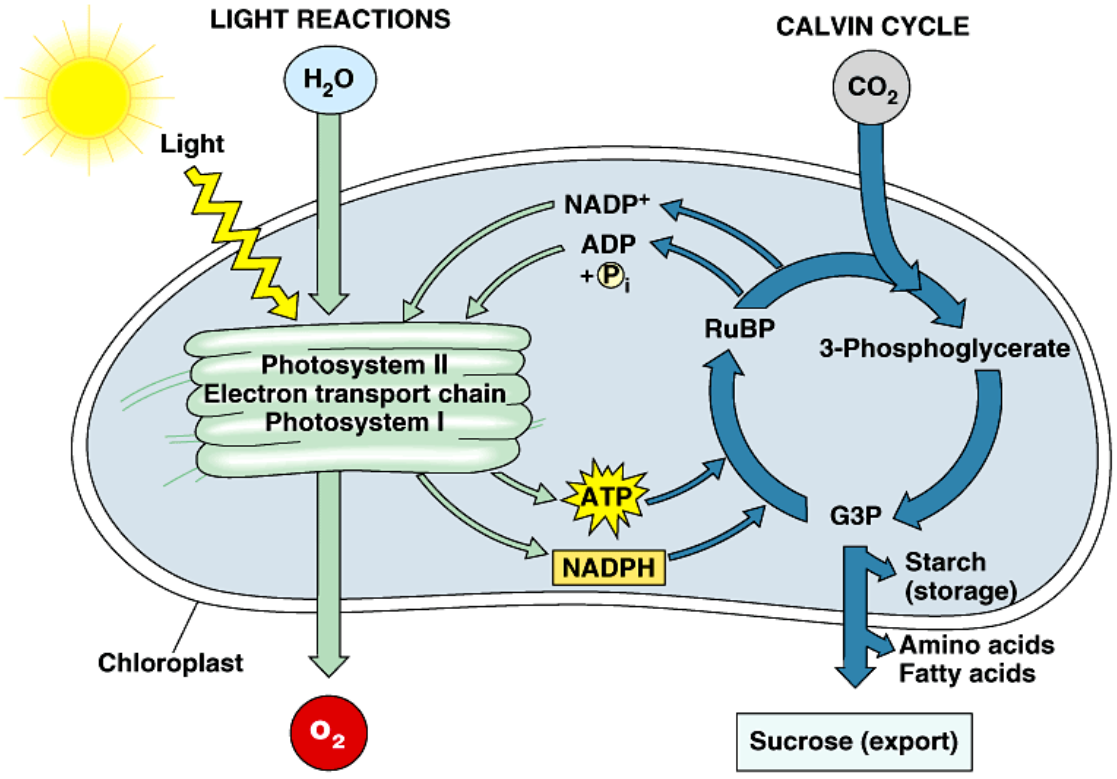


Figure 1-2: The light reactions and the Calvin Cycle of the photosynthesis. *RuBisCO catalyze the rate-limiting step of the Calvin cycle. ATP and NADPH are produced through the light reactions. Illustration from http://mrskingsbioweb.com.*

1.2 *Chlamydomonas reinhardtii*

1.2.1 The model organism *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii (hereafter referred to as *Chlamydomonas*) is a eukaryotic green alga, commonly found in soil and fresh water (Figure 1-3). It is unicellular, 10 μm in length and 3 μm in with, and with two flagellae (Merchant et al., 2007). The cell harbours three genomes; the nuclear (~ 121 Mb), the mitochondrial (~15.8 kb) and the chloroplast genome (~ 200 kb). The *Chlamydomonas* wild type mutant laboratory strain CC137 (mt+) originates from an isolate made in 1945. The alga is a model organism, used for research on different biological processes, including photosynthesis, protein synthesis, stress responses and flagella motility (Harris, 2001; Merchant et al., 2007).

It has several features making it a popular research target. *Chlamydomonas* can grow both photosynthetically and with acetate as sole carbon source. Because non-photosynthetic mutants are viable, these have been used to study the photosynthetic process. Its capability of growing photosynthetically, heterotrophically and mixotrophically also makes it possible to control its life cycle by nitrogen and light. Alternating periods of light and dark synchronizes cell division (Lien & Knutsen, 1979), and nitrogen starvation triggers sexual propagation. Under ideal growth conditions the alga reproduces only through mitosis. The progeny inherit chloroplast DNA only from the maternal mating type parent (Sager & Ramanis, 1973) . Since *Chlamydomonas* species are normally haploid, the effects of mutations are seen immediately without further crosses. Under nitrogen starvation haploid gametes develop. There are two mating types, mt (+) and mt (-), which can fuse to form a diploid zygote that serves as a dormant form of the species in the soil. This zygote can under favourable conditions release four flagellated haploid cells by meiosis.

There are also other advantages of using *Chlamydomonas* as a model organism. It is easy to maintain and propagate under laboratory conditions, needing only an

inexpensive salt medium to grow and it can form clear colonies on agar plates. In 1988, it was developed a chloroplast transformation system for *Chlamydomonas* based on micro projectile bombardment (Blowers et al., 1989; Boynton et al., 1988). It allows for exogenous reporter genes and mutated versions of endogenous genes to be stably introduced into the chloroplast genome via homologous recombination. The chloroplast of *Chlamydomonas* is one of few chloroplasts that can be efficiently transformed, and this has made the chloroplast of *Chlamydomonas* a widely used model system for the study of photosynthetic processes.

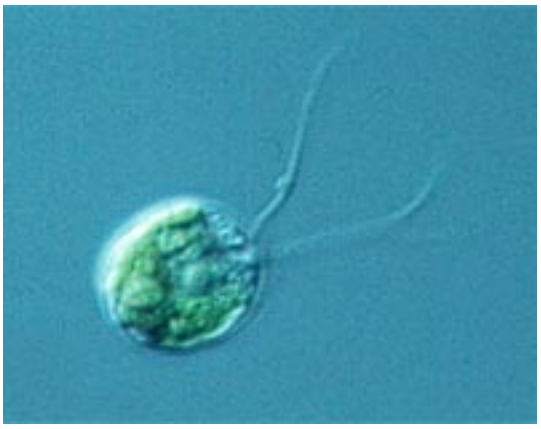


Figure 1-3: The unicellar Chlamydomonas. *It is 10 µm in diameter and swims with two flagella. Picture from http://www.terradaily.com.*

1.2.2 The chloroplast genome of *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii contain a single cup-shaped chloroplast that occupies nearly 40% of the cell volume. Each chloroplast of *Chlamydomonas* contains 50 to 80 copies of the genome. The size of the chloroplast genome is 203 395 bp, and its full sequence was determined in 2002 (Maul et al., 2002) (Figure 1-4). It consists of two inverted repeats (IR) of 21.2 kb each and two in between areas of ~81 kb and ~78 kb (Maul et al., 2002). The inverted repeats are believed to originate from a transposition event, and make the genome able to undergo "flip-flop" recombination between the repeats (Aldrich et al., 1985). The genomes are found in the chloroplast as monomeric and dimeric linear and circular genomes (Maul et al., 2002). As in most

plastid genomes the majority of its genes code for products involved in gene expression and the photosynthetic apparatus.

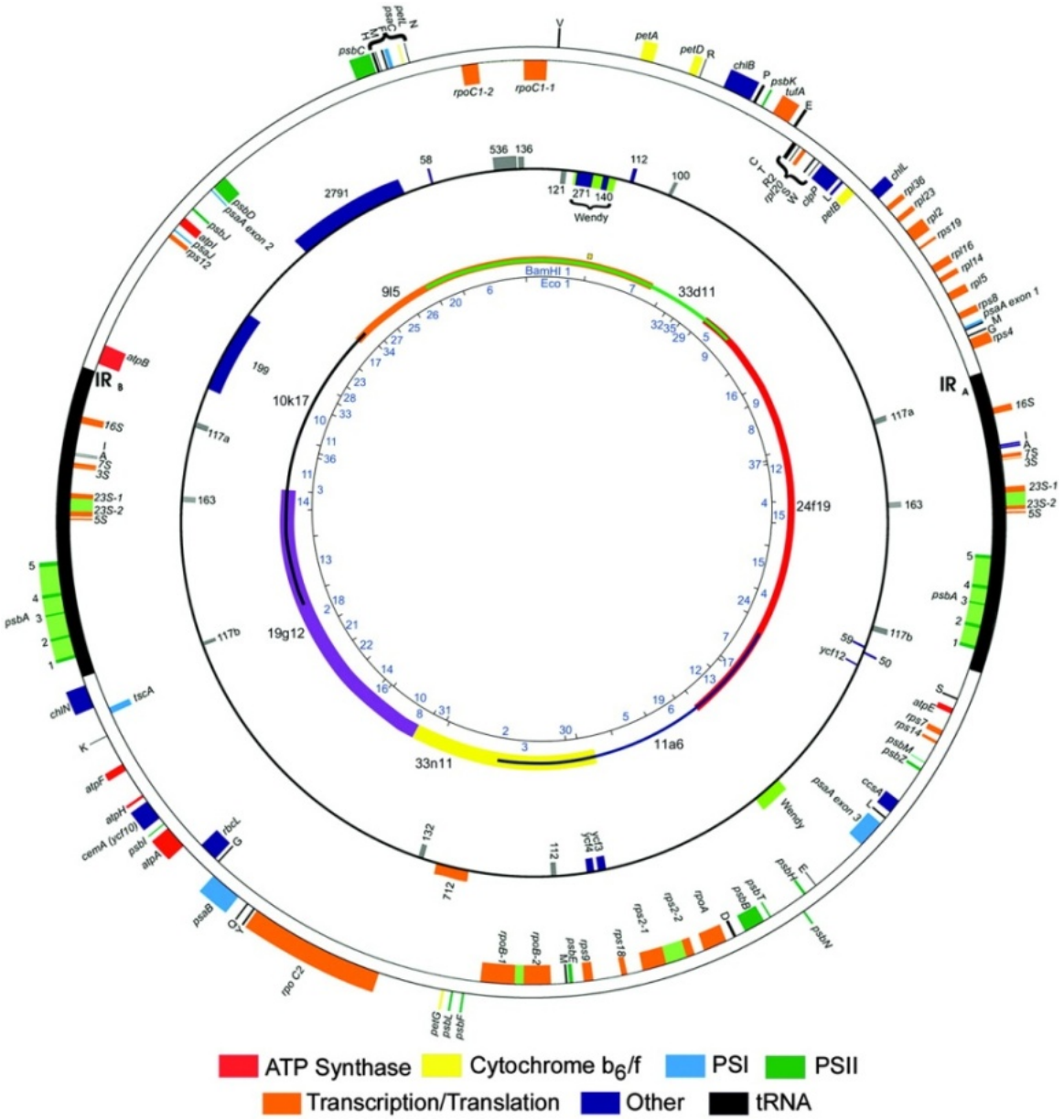


Figure 1-4: The plastid chromosome of Chlamydomonas. *The inner circle shows BamHI and EcoRI restriction fragments. The second concentric circle indicates seven overlapping BAC clones that span the genome. The third circle shows genes and ORFs of unknown function, including those for which disruption experiments were unsuccessful. The outer circle shows genes of known or presumed function, with sequenced or hypothesized introns shown in olive green. Genes are color coded by their function, as shown at bottom (Maul et al., 2002).*

The *Chlamydomonas* chloroplast genome distinguishes itself from other chloroplast genomes in several ways. It is encoding only 99 genes (most plastids contain around 130 genes) (Simpson & Stern, 2002), has an atypical organization of genes encoding the RNA polymerase (they are widely separated and contain introns), and more than 20% of the genome is repetitive DNA. The majority of intergenic regions consist of numerous classes of short dispersed repeats (SDRs) that might have structural or evolutionary significance. Phylogenetic reconstruction of changes shows that SDRs proliferate in the *Chlamydomonas* chloroplast genome, but there is also an accelerated rate of gene loss in the *Chlamydomonas* plastid. In contrast to most chloroplast genes of vascular plants, most *Chlamydomonas* chloroplast genes are not organised in polycistronic transcription units (Drapier et al., 1998).

1.3 Chloroplast gene expression

1.3.1 Chloroplast transcription

RNA polymerases

Plastid transcription is performed by two types of RNA polymerases with distinct transcriptional activities. They are termed PEP and NEP.

PEP is a plastid-encoded multisubunit RNA polymerase. It is present in plastid genomes of all types of photosynthetic plants and algae, and is the only active RNA polymerase in the *Chlamydomonas* chloroplast (Lilly et al., 2002). The plastid-encoded RNA polymerase resembles the *E.coli* σ^{70}-RNA polymerase, and is thought to have evolved from the RNA polymerase of endosymbionts (Igloi & Kössel, 1992). The PEP core enzyme comprises four subunits (α, ß, ß´ and ß″) which are homologues to the bacterial core subunits of RNA polymerase (α, ß and ß´). In bacteria they are encoded by the three genes *rpoA, rpoB* and *rpoC*. The homologous genes in plastids are normally *rpoA, rpoB, rpoC1* and *rpoC2* (the *rpoC* gene split in two) (Igloi & Kössel, 1992). *Chlamydomonas* has an atypical organization of genes encoding PEP (Maul et al., 2002). The *rpoC1* gene is lacking, and *rpoB* is instead split into two genes.

Both the bacterial and the plastid RNA polymerase depend on sigma-factors for promoter specificity (Troxler et al., 1994). The PEP sigma-factors are not encoded in the plastid genomes, but by a small family of nuclear genes and transported into the plastids (Asayama et al., 1996). They have sequence homologies to bacterial sigma factors, and originate from a sigma factor encoded in the endosymbiotic eubacteria. While in most plastids sigma-factors have evolved into different types that can differentiate transcriptional regulation (Allison, 2000) the *Chlamydomonas* chloroplast has only one sigma factor , RPOD, (Bohne et al., 2006) that contributes to general up-regulation of chloroplast transcription in a light-dependent (circadian clock) manner (Carter et al., 2004). PEP transcribes photosynthesis-related genes (Stern et al., 1997), and the sigma-factors make them capable of responding to external factors (e.g. light) and endogenous signals at a transcriptional level.

NEP is a nuclease-encoded single-peptid RNA polymerase. It is present in higher plants, where it transcribes non-photosynthetic housekeeping genes (Hess & Borner, 1999). It is homologous to T3/T7 bacteriophage RNA polymerase, and most likely evolved through a gene duplication of mitochondrial RNA polymerase (also nucleus encoded). NEP is active in proplastids and non-photosynthetic tissue and does not require sigma-factors (Shiina et al., 2005).

Plasmid promotors

PEP and NEP recognize distinct types of promoters.

Standard PEP promoters resemble *E.coli* σ^{70}-type promoters and are characterised by -10(TATAAT) and -35(TTGACA) consensus sequence elements (Hayashi et al., 2003; Sugiura et al., 1998). Positions are relative to start site of transcription. The strength of the promoters varies considerably, and is probably determined by similarity of the promoter elements to consensus sequences. It is the most common promoter in higher plants. In *Chlamydomonas* chloroplast rRNA is transcribed by this sort of promoter (Klein et al., 1992). Protein-coding genes are transcribed by another type of promoter (Salvador et al., 2004a). It lacks the -35 consensus motif, but

includes a palindrome sequence element TATAATAT around position -10 (Klein et al., 1992). A third promoter type is identified in spinach and *Chlamydomonas*. It is thought to be an internal promoter of a subpopulation of chloroplast tRNA genes.

NEP promoters are found in higher plants. Most NEP polymerases recognise an YRTA-motif which is similar to mitochondrial Ia promoters (Hess & Borner, 1999; Weihe & Borner, 1999). In several genes of higher plants the NEP promoter has been found upstream of PEP promoters (Hajdukiewicz et al., 1997) . NEP polymerase is the most active polymerase during plastid development, when photosynthesis related genes are largely silent (Baumgartner et al., 1993). PEP polymerase takes over as the most active later in plant development. Some genes can therefore be transcribed by different polymerases at different stages of plant development (Klein et al., 1994; Magee & Kavanagh, 2002).

Transcriptional regulation

Unlike prokaryotes, chloroplast genes are rarely regulated individually at a transcriptional level (Bollenbach et al., 2004). Transcription can be regulated globally by changes in genome copy number (Simpson & Stern, 2002), by changes in DNA supercoiling in plastid nucleoids (Salvador et al., 1998), by intracellular relocation of plastid nucleoids and by phosphorylation of RNA polymerases (Shiina et al., 2005). *Chlamydomonas* chloroplast transcription has been found to be globally up-regulated in light and can exhibit a circadian oscillation (Salvador et al., 1993b). Transcription also can be differentially enhanced by illumination (Klein & Mullet, 1990) and both PEP and NEP promoters can be regulated by extra *cis*-elements and various nuclear-encoded transcription factors (Shiina et al., 2005).

1.3.2 Post-transcriptional processing.

In plastids, primary transcripts undergo a series of mRNA maturation steps. These include RNA editing, intercistronic processing, intron splicing and processing of the 5' and 3' ends. These modifications prepare the transcripts for translation. Post-transcriptional processing can be used by the cell for protein level regulation. Some of these RNA processing steps exhibit prokaryotic features that have been retained from the eubacterial ancestor of present-day plastids, but they have also obtained features normally associated with eukaryotic nuclear genomes (Herrin & Nickelsen, 2004; Monde et al., 2000).

RNA editing

RNA editing occurs in chloroplasts of higher plants and in eukaryotic nuclear genomes (Freyer et al., 1997), but not in chloroplasts of algae or in prokaryotes (Barkan & Goldschmidt-Clermont, 2000). RNA editing activity in plastids is responsible for specific Cytosine-to-Uracil conversions in the nucleotide sequence of transcripts prior to other post-transcriptional processes. Examples of Uracil-to-Cytosine conversions have been found in ferns and hornworts (Yoshinaga et al., 1996). Editing mostly affects protein-coding sequences, but has also been found to occur in non-coding transcribed regions and structural RNAs. The change in the coding sequence leads to a changed translated protein, often with higher similarity to the corresponding non-plant homologues. RNA editing can also affect initiation or stop codons, or binding sites for proteins necessary for initiation of translation (Esposito et al., 2001). The editing sites have sequences (*cis*-acting elements) of ~15 nucleotides in their immediate upstream region. These elements are recognized by site-specific proteins (*trans*-acting editing factors), encoded in the plant nuclear genome (Miyamoto et al., 2004). RNA editing activity in plastids is probably not a regulatory mechanism of gene expression, but has earlier in evolution given enhanced genetic variation at RNA level (Tillich et al., 2006).

Intercistronic processing

An mRNA is monocistronic when it contains genetic information to translate only a single protein. Polycistronic mRNAs carry the information of several genes, which are translated into several proteins. As in eubacteria, most plastid genes are organized in polycistronic transcription units (operons). *Chlamydomonas* is an exception from this, with mostly monocistronic units (Rochaix, 1996). In plastids, most polycistronic precursor transcripts are processed into monocistronic units by specific endonucleolytic cleavage (Herrin & Nickelsen, 2004). It is also possible for some polycistronic chloroplast mRNAs to be directly translated (Carpousis et al., 1989). As genes in the same operon are transcribed together, the same promoter controls their transcription. The rate of processing reactions can vary significantly during plant development (Riesmeier et al., 1994), and processing can, therefore, be considered as a possible regulator mechanism of gene transcription.

Intron splicing

While absent in bacteria, introns are present in a number of plastid-encoded genes. In higher plants ~17% of the plastid genes contain introns and most of them belong to intron group II (Plant & Gray, 1988). Sequencing of the *Chlamydomonas* chloroplast genome has confirmed that it contains few introns. They are located in the *psbA*, *psaA* and 23S rRNA genes (Maul et al., 2002). The introns belong to both group I (*psbA* and 23S) and group II (*psaA*). The group II introns are spliced in *trans* (exons joined from two different transcripts). It is likely that these introns need *trans*-acting splicing factors (Herrin & Nickelsen, 2004). The group I introns are spliced in *cis* and are self-splicing (Cech, 1990) but probably use *trans*-acting proteins to promote the splicing (Li et al., 2002). Splicing rate of the *psbA* introns has been found to be increased by light. As group I intron ribosomes require cations for folding and catalysis, this induction is probably electron transport dependent (Deshpande et al., 1997).

Processing of the 5' and 3'ends

Most plastid transcripts undergo specific processing at the 5'and 3'ends. The role of 5'mRNA processing is still unclear, but for the *psbA* mRNA, it has been linked to

coupling to the ribosomes (Bruick & Mayfield, 1998). Some *Chlamydomonas* transcripts, e.g. *psbD* and *atpB*, can be found in the cell as two different forms. A low abundant precursor form and a shorter, predominant form, that is most likely generated by a 5'processing event (Anthonisen et al., 2001). 3'end processing is done at the 3'terminus to remove nucleotides added during transcription. This is done by ribonucleases and is for most chloroplast mRNAs essential for transcript stability (Drager et al., 1996; Stern et al., 1989). A general feature in plastid-encoded mRNA is the presence of inverted repeats in the 3'UTR that fold into stem-loop structures. These structures resemble transcriptional terminators in bacteria, but are probably not terminator signals. They are likely 3'end-prosessing signals and preventing 3' to 5' exonuclease degradation (Rott et al., 1998). 3'prosessing creates an mRNA terminating in a stem-loop structure. In *Chlamydomonas*, 3'end maturation of the *atpB* mRNA starts with an endonucleolytic cleavage at an AU-rich site located ~10 nucleotides downstream of a stem-loop structure. This cleavage is followed by exonucleolytic trimming to generate the mature 3'end (Stern & Kindle, 1993).

1.3.3 Transcript Degradation

The level of mRNAs in the chloroplast is determined not only by its synthesis, but also by the decay rate. While the 3'regions of eukaryote transcripts have been found to be primary modulators of mRNA longevity, in bacterial and organelle mRNAs the essential determinants of transcript longevity seem to be located mostly in the 5'UTR. Plastid mRNAs are protected from degradation by *cis* and *trans*-acting elements. The transcripts are rapidly degraded by ribonucleases when the protection from these elements is removed. Ribonucleases are divided into exonucleases that start degrading from either the 3'end or the 5'end, and endonucleases that start degrading inside the sequence.

mRNA stability is measured in mRNA half-life. Half-life is the time required for half the initial amount of RNA to disappear (Monde et al., 2000). Different mRNAs within the same cell have distinct lifetimes that vary between species. Chloroplast mRNA is more resistant to degradation than bacterial mRNA. The half-life of bacterial transcripts range from seconds to half an hour, while plastid transcripts have half-life of several hours (Salvador & Klein, 1999).

The degradation machinery in chloroplasts has some similarity to that of bacteria. Plastid homologues to the bacterial endonuclease RNaseE that cleave AU-rich elements have been found (Linchao et al., 1994). But as indicated by the great difference in longevity of transcripts, there also are differences. In bacteria the presence of a hairpin structure at the 5'end of mRNA has been found sufficient to maintain the longevities of the transcript. This is supposedly because the hairpin secondary structures hinder the endonuclease RNaseE from doing the initial nucleolytic attack. In chloroplasts, the secondary structures of their 5'end has been found insufficient to maintain the long lives of the mRNAs, and it has been shown that nucleus-encoded protein factors are required for transcript stability in the chloroplast (Kuchka et al., 1989; Nickelsen & Kück, 2000; Sieburth, et al., 1991).

Rates of transcription in mature chloroplasts are relatively constant despite external or developmental signals. It is therefore thought that gene expression is mainly regulated at a post-transcriptional level (Mayfield et al., 1995). Chloroplast mRNA has relatively long lives, and regulation of decay makes the cell capable of rapid change in protein level. Down-regulation of mRNA degradation can quickly result in higher protein levels because translation can start immediately from an already existing mRNA pool. Up-regulation of mRNA decay will also affect mRNAs already transcribed, and thus more efficiently hinder proteins synthesis. It is beneficial for the chloroplast to be capable of making rapid changes in protein synthesis levels in response to external factors (e.g. light), and RNA decay rate changes are therefore thought to be the mature chloroplast's main response to environmental changes.

The importance of the 3'UTR for mRNA stability

The stem-loops at the 3'ends of mature chloroplast mRNAs are necessary for stability of the transcripts. The stem-loops are increasing the stability of the mRNA by sterically hindering 3' to 5'exonucleases attack and blocking polyadenylation (Lisitsky, et al., 1996). The stem-loops must be removed by endonucleolytic cleavage (performed by CSP21a, CSP41b and possibly an RNaseE homolog) before 3' to 5'exonucleolytic degradation can occur (Bollenbach et al., 2004).

In *Chlamydomonas petD*, *psbA* and *rbcL* mRNA the sequence-unspecific endonuclease CSP41a has been found to cleave within the 3'stem-loop. This initiates mRNA decay (Bollenbach, et al., 2003). In the C*hlamydomonas rbcL* mRNA there are found two *cis*-acting elements in 3'UTR stem-loops, which independently are sufficient to maintain mRNA stability (Goldschmidt-Clermont, 2007). The stabilizing element is likely the structure of the stem (Gruissem W. et al., 1986).

In *Chlamydomonas* one nucleus-encoded regulator protein (crp3) that targets the 3'UTR has been identified. The *trans*-acting factor has several plastid mRNA targets (Levy et al., 1999). The 3'UTR of *rbcL* mRNA has not been found to be important for light-dependent regulation.

3’ to 5’degradation in chloroplasts can probably be poly(A) tail independent, performed by RNaseII and PNPase (Bollenbach et al., 2004), but the best defined pathway starts with polyadenylation. Poly(A) tails promote transcript degradation in bacteria and chloroplasts. In mitochondria polyadenylation can promote both degradation and enhanced stability. The poly(A) tails target the RNAs for exonucleolytic 3’ to 5’end degradation by PNPase which has a poly(A) RNA binding site (Hayes et al 2003). Polyadenylation sites in plastids are frequently found within the coding region (Klaff, 1995). In *Chlamydomonas* poly(A) tails have been detected on mRNA, tRNA and rRNA (Komine et al., 2000). They have been reported to destabilize the *atpB* gene (Komine et al., 2002).

The importance of the 5’UTR for mRNA stability

Unlike all prokaryotes investigated to date, chloroplasts contain a 5’ → 3’ degradation pathway (Drager et al., 1998). The ribonucleases responsible for this decay have not yet been identified, but analysis of the effect of mutations in 5’UTR sequences on mRNA longevity has revealed that plastid 5’UTRs can be determinants of mRNA stability. In *Chlamydomonas*, insertion of a poly(G) cassette, which impedes movement of exoribonucleases along RNA molecules in the 5’UTR of *petD* (Drager et al., 1999) and *psbB* (Vaistij et al., 2000) genes protected against decay. These facts imply the existence of 5’ to 3’exonucleases. The 5’UTRs of chloroplast mRNAs have been found to harbour stem-loops, similar to the stabilizing stem-loops in the 3’UTR, but it has not been found evidence that it is the secondary structure of the 5’ stem-loops that define the regulatory qualities of the 5’UTR. The stabilizing elements are likely *cis*-acting sites recruiting sequence specific *trans*-factors (Suay et al., 2005). There have not been found any *cis*-acting consensus sequences in the 5’UTRs of plastid transcripts. This implies that *trans*-acting factors stabilize specific transcripts, and can be used for individual regulation of different transcripts.

***Trans*-acting factors**

Analysis of nuclear mutants has revealed several loci that affect mRNA stability. These nuclear loci encode proteinaceous *trans*-acting factors which enhance the stability of the mRNAs. Most of these stability factors have been found to function

via association with specific sequences in 5'UTRs. Direct evidence is missing for binding between proteins and stabilizing *cis*-acting elements, but there have been identified proteins that complement RNA stability mutants and proteins that bind to mRNAs 5'UTR (Suay et al., 2005). Examples of these are the *Chlamydomonas* genes *mbd1*, *mcd1*, *mbb1* and *nac2* that are found to stabilize the mRNAs of *psbD*, *petD*, *psbB* and *psbD* respectively (Boudreau et al., 2000; Drager et al., 1998; Nickelsen et al., 1994; Vaistij et al., 2000).

The amino acid sequences of *mbd1* and *mbb1* revealed that both proteins harbour a protein-protein interaction motif called TRP (tetratriconpeptide motif). In vascular plants a related motif, PPR (pentatriconpeptide motif), has been found in nuclear factors which process chloroplast mRNA. Homologues of this PPR domain have been found in several nuclear-encoded *Chlamydomonas* genes (Nickelsen, 2003). An intraction of nuclear-encoded proteins with other proteins in the cell has been further indicated by finding the mbd1 and mbb1 proteins in high molecular weight complexes (Boudreau et al., 2000; Vaistij et al., 2000).

It is possible some of the *trans*-acting factors are redox-carrying proteins, and thus are influenced by light. Difficulties in finding these *trans*-acting factors might be due to the requirement for particular secondary structures that may be hard to establish *in vitro* (Suay et al., 2005).

Cis-acting factors

The capability of *Chlamydomonas* chloroplast to be efficiently transformed has made it possible to identify the targets of nuclear *trans*-acting factors in the alga. The *cis*-acting stability determinants have mostly been found in the 5'UTR of transcripts. Exceptions are the *cis*-loci in the coding region of *atpA* mRNA and the nuclear-encoded protein crp3's targets in several chloroplast 3'UTRs (Levy et al., 1999). In *petD* mRNAs there has been found an 8-nucleotide RNA stability element in a stem-loop structure at the 5'end terminus (Drager et al., 1998; Higgs et al., 1999). In *psbD* transcripts two distinct elements are required for stable accumulation of *psbD* mRNA.

Neither is predicted by computer analysis to form any significant secondary structure. One of these elements is located at the 5'terminal end of the precursor transcript. The other has a location making it a part of the mature mRNA (Nickelsen et al., 1999).

Light dependent regulation of RNA decay

Light can affect both RNA synthesis and degradation in chloroplasts (Thompson & Mosig, 1984). In the chloroplasts of *Chlamydomonas* and tobacco reduced transcription rates and an increase in RNA stability were found in dark (Salvador et al., 1993b; Shiina et al., 1998).

A possible mechanism by which transcript turnover can be regulated by light/ dark is through the concentration of divalent cations. The concentration of stromal Mg^{2+} is found to be significantly higher in light grown leaves than in dark adopted leaves (Bollenbach et al., 2003). The ribonucleases RNaseE, CSP41a, PNPase and RNaseII are known to require divalent-metal ions to perform cleavage. Divalent cation levels can therefore be a control mechanism for light-dependent transcript degradation. In *Chlamydomonas* Mg^{2+} levels has been found to have a destabilizing effect on *psbD* 5'UTR RNA (Nickelsen et al., 1994). It is also possible for chloroplast ribonucleases to be under control of redox conditions, but to date only the mustard p54 chloroplast ribonuclease has been shown to be under this control (Nickelsen & Link, 1993).

Light-dark regulation can also be done through stabilizing/ destabilizing *trans*-acting factors. Rates of degradation have been linked to the redox state in the chloroplast and are thought to involve chloroplast proteins that function as redox carriers (Anthonisen et al., 2001; Salvador & Klein, 1999).

1.4 The 5'end of the *Clamydomonas rbcL* transcript

1.4.1 The *rbcL* 5'UTR affects light-dependent decay rates

The 5'UTR is a modulator of transcript longevity

In studies where the 3'UTRs of the *Chlamydomonas rbcL* transcripts were fused 3' to GUS genes, it was found that the 3'UTRs did not affect rates of degradation (Blowers et al., 1993). It was concluded that *rbcL* 3'UTRs are not important in modulation of decay rates. But the 5'UTRs of *Chlamydomonas rbcL* transcripts were found to induce rapid light-dependent degradation of mRNA when they were fused to the coding region of foreign genes (Salvador et al., 1993a). From this, it was concluded that sequences in the 5'UTR are sites of determinants of transcript longevity.

Element reversing the destabilizing effect of the 5'UTR

The coding region of *rbcL* has a stabilizing sequence in position +329 to +334 (relative to start of transcription) that reverses the destabilizing effect of the 5'UTR (Singh et al., 2001). It has been postulated that there is a protein binding sequence both in the 5'UTR and the coding region, and that these interact either by binding to the same protein, or binding two different proteins that interact, possibly through a larger protein complex (Singh et al., 2001). It has been found that most stabilizing *trans*-acting factors are TPR-proteins with protein-protein interaction sites.

The longevity of rbcL transcripts

WT-*rbcL* transcripts are stable in the light, and have a half-life of 21 hours in the dark, and 3.5 to 5 hours in light. Because of reduced transcript synthesis in the dark this results in constant *rbcL* levels, independent of light/ dark conditions. However, the chimeric GUS genes used in this study do not include the stabilizing element of the *rbcL* coding region, and have a half-life of 4 to 5 hours in the dark and only 20 min in the light when a WT-*rbcL* 5'UTR is used (Anthonisen et al., 2001). RNA from the reporter gene must therefore be isolated in the dark, to avoid light-dependent degradation.

1.4.2 Elements at the 5'end of *rbcL* transkripts

The 5' UTR of the *rbcL* transcript consists of 92 nucleotides. It is predicted to fold into two stem-loop structures (Figure 1-5). The first stem-loop is situated at the 5'end, and consists of 41 nucleotides. The second and smaller stem loop is situated between nucleotides +45 and +66, relative to the start of transcription. The promoter of the *rbcL* gene is a -10 palindromic sequence (TATAATAT) (Salvador et al., 2004b). An enhancer has been found to extend from position +108 to +143. This sequence element is able to increase transcription about 10-fold (Anthonisen et al., 2002).

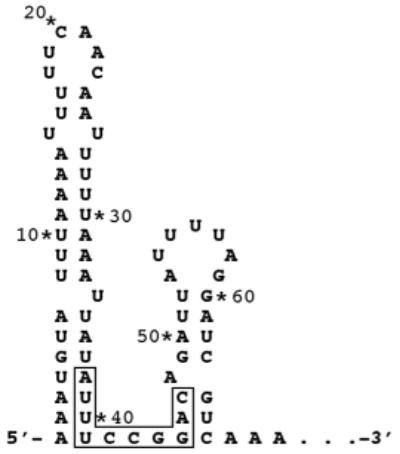


Figure 1-5: The predicted RNA secondary structure of the 69 first nucleotides of the Chlamydomonas rbcL transcript. *The cis-acting stability element (+38 to 47) is boxed. (Figure from Suay et al., 2005).*

A possible light-dependent degradation target

Because the *rbcL* 5'UTR were found to induce light-dependent degradation when fused to the coding region of foreign genes it has been postulated that there is a light-dependent degradation target within the first 63 nucleotides of the 5'UTR. Previous mutation analyses has shown that the postulated degradation target is likely to be either a specific sequence between nucleotide +20 and +41, or the secondary structure of the first stem loop (endonuclease target) (Singh et al., 2001). However, the first stem-loop has been found to have no effect on the transcript stability, and no specific destabilizing sequence has been found. It is possible that light dependent RNAase is responsible for enhanced decay upon illumination. The stabilizing *trans*

element postulated to bind/ be stabilized by the *cis*-acting sequence in positions +329 to +334 may or may not be redox-dependent, as it also has a stabilizing effect in the dark.

A light-independent cis-acting sequence

Various mutations in the first 64 nucleotides of the transcript have been found to destabilize the chimeric *rbcL*: GUS transcript to a degree where no transcript is detectable by northern analysis, even when RNA is isolated in the dark. Chimeric genes with destabilizing mutations are estimated to have a half-life of well below 5 min (Salvador et al., 2004b). These studies have led to the conclusion that there is a *cis*-acting stabilizing element in the *rbcL* 5' UTR, postulated to work through binding of a proteinaceous *trans*-factor, hindering nuclease attack. Mutation studies have shown that transcripts with mutations in nucleotides +38 to +47 or mutations that disturbs the secondary structure of this sequence renders the transcript highly unstable (Anthonisen et al., 2001). This places the *cis*-acting element in the area between the stem-loops, and the lower part of the stems (Figure 1-5). It also implies that the *cis*-acting element is recruiting a sequence specific *trans*-acting factors that also depend on a specific secondary structure. There has not yet been found any sequence similar to the 10-nucleotide RNA-stabilizing element in other chloroplasts (Anthonisen et al., 2001).

Rates of transcription have been investigated for mutated chimeric *rbcL* 5'end: GUS transcripts with point mutation in positions + 40 and +46 (Anthonisen et al., 2001). Mutations were not found to affect transcription. The effect of light has also been investigated for the chimeric *rbcL* 5'end: GUS transcripts with modified *rbcL* sequences in positions +38 to +47 (Anthonisen et al., 2001). It was found that the mutated transcripts still showed the typical light/ dark regulation of abundance, suggesting the dark/ light regulated mechanism of transcript destabilization is distinct from the RNA decay mechanism involving the *cis*-acting element between nucleotides +38 to +47.

1.5 The scope of this study

This study is part of a project in which the 5'UTR of the *Chlamydomonas rbcL* gene is analyzed for its effect on transcript stability. The transcript folds into a 5'terminal stem-loop structure, closely followed by a smaller stem-loop. The two stem-loops are spaced by three unpaired nucleotides (figure 1-6).

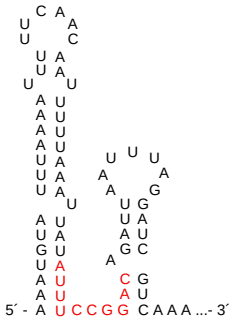


Figure 1-6: The 5'UTR of a Chlamydomonas rbcL transcript. *Nucleotides in positions +1 to +69 relative to start of transcription. The cis-acting stability element (nucleotides +38 to +47) shown in red.*

A series of mutation studies has led to the conclusion that the sequence and conformation of a 10-nucleotide element in the *rbcL* 5'UTR is essential for stabilizing chimeric GUS transcripts (figure 1-6) (Anthonisen et al., 2001). The presence of two stem-loops has been found necessary to give this element its secondary structure.

The role of the second stem-loop remains uncertain, but is being investigated through various mutation studies. In this study the upper sequence of the second stem-loop (positions +50 to +63 relative to start of transcription) was changed in a way that kept the secondary structure and the size of the stem-loop, to investigate the importance of the nucleotide sequence.

It has previously been shown that adding up to 8 nucleotides to the 5'terminus of the *rbcL* transcript does not significantly affect the stability of chimeric GUS transcripts, while adding 10 nucleotides or more abolish transcript accumulation completely. A second modified *rbcL* 5'UTR: GUS construct was made as part of this study where 9 nucleotides were added 5' to the transcript.

2. MATERIALS AND METHODS

2.1 *Escherichia coli*: bacterial strains, media and methods

2.1.1 Growth of *E.coli*

Recombination-deficient strain *E.coli* TB1 was used for cloning. The strain is not ampicillin resistant.

E.coli was grown in liquid LB medium (10 g tryptone/ l, 5 g yeast extract/1 and 10 g NaCl/1) or LB plates (LB with 1.5% agar) at 37°C.

2.1.2 Preparation and transformation of competent *E.coli* cells.

E.coli cells were made competent by $CaCl_2$ treatment (Sambrook & Russell, 2001).

Frozen competent cells were melted on ice prior to adding DNA. Heat shock (42°C for 90 seconds), followed by immediate cooling on ice was used for transforming the cells (Sambrook & Russell, 2001). Transformants were selected using ampicillin-containing (60 μg/ml) LBA plates.

2.1.3 Plasmid isolation from *E.coli*

Mini-prep

For small-scale plasmid isolation mini-preps were performed according to protocol (Sambrook & Russell, 2001). Transformed cells where grown overnight, to stationary phase, in ampicillin-containing (60 μg/ml) LB medium before isolation. Plasmid isolation from 1.5 ml cell culture gives an expected yield of 1 to 3 μg DNA.

Maxi-prep

For large-scale plasmid isolation, a maxi-prep was performed. The CsCl density gradient centrifugation method was used according to the protocol (Sambrook & Russell, 2001). Transformed cells where grown overnight in ampicillin-containing

(60 µg/ml) LB medium before isolation. Plasmid isolation from 100 ml cell culture yields about 150 to 400 µg of DNA.

2.2 *Chlamydomonas reinhardtii*: algal strains, media and methods

2.2.1 *Chlamydomonas reinhardtii* strain

The *atpB*-deficient *Chlamydomonas reinhardtii* mutant strain, CC-373 (ac-u-c-2-21 mt+), was obtained from the *Chlamydomonas* Genetics centre at Duke University, NC, USA. The mutant strain has a 2.5 kb deletion of the 3' half of the *atpB* gene and a portion of an adjacent inverted repeat (Figure 2-1) (Blowers et al., 1989). Because the *atpB* gene is encoding the chloroplast ATP synthase β-subunit the strain is ATP synthase-deficient. This makes the mutant alga light-sensitive, non-photosynthetic and acetate-requiring. CC-373 has been widely used as a recipient for chloroplast transformation experiments.

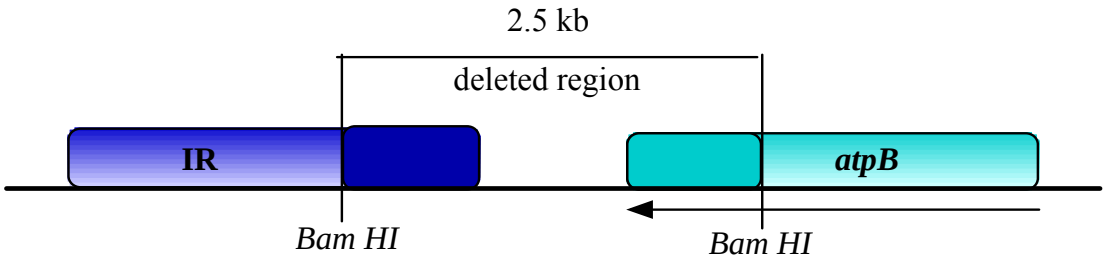


Figure 2-1: The 2.5 kb deletion in CC-373 and flanking regions. *The deletion removes a part of the atpB gene and inverted repeat (IR) between the two BamHI restriction sites. The atpB gene is transcribed from right to left.*

2.2.2 Media for growing *Chlamydomonas reinhardtii*

The *Chlamydomonas* transformants have restored *atpB* genes (re-established photosynthetic capacity), and were grown in HS (high salt) medium (Sueoka, 1960). The *atpB*-deficient *Chlamydomonas* mutant was grown in HSHA (high salt high acetate) medium, which is HS medium supplemented with potassium acetate (2.5 g/l). Media for plates were made by adding 1.5% agar to HS or HSHA.

2.2.3 Preparation of *Chlamydomonas reinhardtii* cells for transformation

Before transformation CC-373 was grown in liquid HSHA medium (Sueoka, 1960). The cultures were diluted in fresh medium every second day to maintain exponential growth. Because the non-photosynthetic mutant is light sensitive it is kept under low-light conditions. Immediately before transformation cells were plated onto HSHA agar plates.

2.2.4 Transformation of *Chlamydomonas reinhardtii* chloroplast

The DNA was precipitated onto 0.6 µm gold particles, and shot by micro projectile bombardment into the agar-plated mutants (Blowers et al., 1989; Boynton et al., 1988). This was done using the particle delivery system (PSD-1000/He; Bio-Rad), with helium gas at a pressure of 1300 psi (Figure 2-2). After shooting, the agar plates were kept in the dark to allow for recombination to occur between the DNA and the genome chloroplast. Transformants (restored *atpB* gene, capable of photosynthetic growth) were selected by transferring the algae to HS agar plates and exposing them to bright light.

Figure 2-2: The PSD-1000/He Particle Delivery System. *An elemental particle of heavy metal is coated with plasmid DNA and shot by micro projectile bombardment into the chloroplast of the agar plated cells. Picture from: http://en.wikipedia.org/wiki/Gene_gun.*

2.2.5 Growth conditions for transformed *Chlamydomonas reinhardtii*

After transformation the photosynthetic algae were grown on HS agar plates under continuous light. Six colonies were picked, and grown in 200 ml liquid HS medium under continuous light. The cultures were then transferred to 100 ml tubes, placed into a 32°C water bath, and grown under continuous light and bubbling with 2% CO_2–enriched air to improve the growth rate. These cultures were used for DNA isolation. Before RNA isolation the cultures were grown in a water bath in alternating 12 hour dark/ 12 hour light cycles to synchronize the cells life cycles. Total RNA was purified at the end of the dark cycle (11 h) because *Chlamydomonas* RNA levels are highest in the dark. Upon illumination transcripts are degraded (Salvador et al., 1993b).

2.2.6 DNA isolation from *Chlamydomonas reinhardtii*

DNA isolation was performed according to protocol (Blowers et al., 1989).

2.2.7 RNA isolation from *Chlamydomonas reinhardtii*

RNA isolation was performed according to protocol (Sambrook & Russell, 2001).

2.3 Oligonucleotides and Plasmids

2.3.1 Oligonucleotides

Two pairs of synthetic, complementary, single strained oligonucleotides (Figure 2-3) were obtained from MWG Biotech AG. After annealing, both double-stranded sequences had one blunt end and one sticky end with a four-nucleotide overhang (GGCC). The sticky ends are complementary to a *BspEI* restriction site. Sticky ends are easier to ligate and decide the orientation the sequences are ligated into the DNA.

A) Oligonucleotide +9

```
   9 nucleotide addition
5'-AGTATACATAAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTT     - 3'
3'-TCTTATGTATTTACATAAATTTTAAAAAGTTGTTAAAAATTTAATATAAAGGCC- 5'
                                                    overhang
```

B) Oligonucleotide M

```
  overhang
5'-CCGGACAGGCCGCCCCGAGGCCGTCAAAAGAAGTTACATTTATTTATATAAGAT-3'
3'-    TGTCCGGCGGGGCTCCGGCAGTTTTCTTCAATGTAAATAAATATATTCTA-5'
       changed nucleotides
```

Figure 2-3: Sequences of the annealed oligonucleotides. *A) Oligonucleotide +9 with the 9 nucleotide addition in red and BspEI-overhang in green. B) Oligonucleotide M with changed nucleotides in red and BspEI-overhang in green.*

Oligonucleotide +9

The annealed oligonucleotide +9 contains the first 41 base pairs of the 5'UTR in the *Chlamydomonas rbcL* gene (position +1 to + 41 relative to start of transcription). In the 5'end the construct has an addition of nine base pairs (5'-AGTATACAT- 3'). The 3'end of the construct has a four-nucleotide overhang, complementary to the *BspEI* restriction site in the *rbcL*-5'UTR (Figure 2-3A).

Oligonucleotide M

The annealed oligonucleotide M contains 50 base pairs from the 5'UTR of the *Chlamydomonas rbcL* gene (position +46 to +96 relative to start of transcription) with changes in the base pairs in positions +50 to +62. The sequence 5'-AUUUAUUUUAGGAU-3' is replaced with 5'-GGCCGCCCCGAGGC-3'. This alters the nucleotide sequence of the second stem-loop in the 5'UTR of the transcript. The 5'end of the oligonucleotide has a four-nucleotide overhang, complementary to the *BspEI* restriction site in the *rbcL* 5'UTR (Figure 2-3B).

2.3.2 Plasmids

Plasmid +19/SK+

Plasmid +19/SK+ (~5.2 kb) contains a chimeric *rbcL*: GUS sequence (2.2 kb) cloned in between the *XhoI/ XbaI* sites of pBluescript SK+ (Strategene) (figure 2-4). The *rbcL* region consists of 229 base pairs from the 5'end of the *Chlamydomonas rbcL* gene (positions -70 to + 157 relative to start of transcription). This 5'end region is fused 5' to the coding region of the bacterial *uidA* (GUS) reporter gene (Figure 2-4).

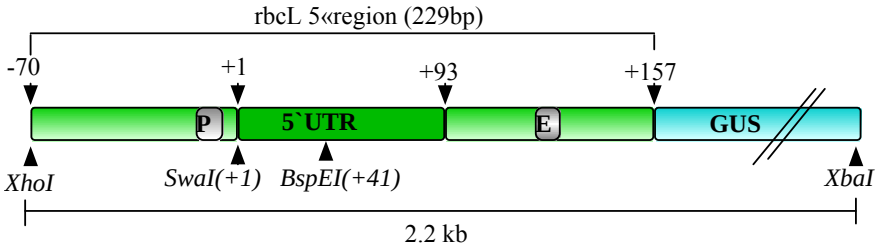


Figure 2-4: The chimeric rbcL: GUS gene in plasmid +19/SK+. *Selected restriction sites are shown below the drawing, and positions above the drawing. The position +1 indicates the transcription start site, and the 5'UTR is located between +1 and +93. P indicates the rbcL promoter and E is the enhancer sequence.*

The *rbcL* 5'UTR harbours a *SwaI*, restriction site in position +1 and a *BspEI* site in +41 relative to start of transcription. The plasmid is encoding ampicillin resistance as selectable marker.

Plasmid +93 (EcoRV)/SK+

Plasmid +93 (*EcoRV*)/SK+ (~3 kb)contains a *rbcL* 5' sequence (nucleotides in positions -70 to +93 relative to start of transcription) cloned into the pBluescript SK+ *XhoI / EcoRV* restriction sites (Figure 2-5).

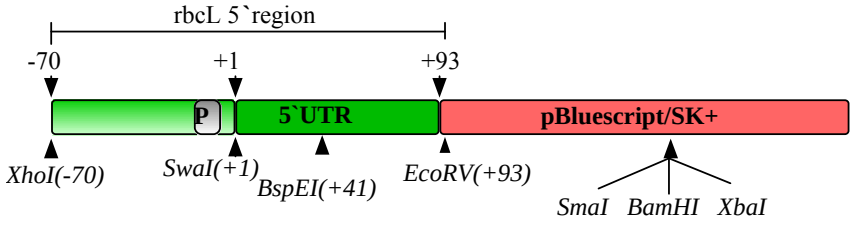


Figure 2-5: The chimeric rbcL: atpB sequence in plasmid +93 (EcoRV)/SK+. *Selected restriction sites are shown below the drawing, and positions above the drawing. The position +1 indicates the transcription start site. P indicates the position of the rbcL promoter.*

The pBluescript SK+ sequence harbours *SmaI*, *BamHI* and *XbaI* sites. The *rbcL* enhancer sequence is not included in this construct. The plasmid is encoding ampicillin resistance as selectable marker.

Plasmid pCrc32

Plasmid pCrc32 (~11 kb) is based on the pUC8 plasmid. It contains two regions homologous to sequences in the chloroplast genome of *Chlamydomonas*, including the complete *atpB* gene and a part of the inverted repeat (IR) region (Figure 2-6). These regions are corresponding to the deleted sequence and the areas flanking the deletion in mutant algae CC-373, and make transformation of the mutant algae possible by homologous recombination.

pCrc32 containes a cimeric gene [*atpB* 5'end: GUS: *psaB* 5'end] cloned in between the *atpB*-gene and the IR (Figure 2-6). The *atpB* 5'region and the GUS gene are flanked by *XhoI*/ *XbaI* restriction sites. The IR region contains a *BspEI* and a *SwaI* restriction site. The plasmid is encoding ampicillin resistance as a selectable marker.

Figure 2-6: The chimeric rbcL: GUS: psaB gene and flanking areas in plasmid pCrc32.
XhoI and XbaI restriction sites are shown below the drawing.

2.4 DNA methods

2.4.1 Oligonucleotide annealing

Complementary oligonucleotide strands were annealed by mixing equal amounts (500ρmol each) of the complementary sequences, before heating to 100°C for 2 two minutes followed by a cool down of 30 min. After annealing, the 5'ends were phosphorylated using 1 µl T4-polynucleotide kinase (10 u/µl), in the presence of 5 µl ATP (10 mM, pH7) and 5 µl PNK buffer (10X) (Fermentas, Life Science) in a total volume of 50 µl. The concentration of the resulting dsDNA fragments was determined by calculation from the oligonucleotide data, supplied by MWG Biotech AG.

2.4.2 Restriction enzymes

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

2.4.3 Agarose gel electrophoresis

A 1% or a 1.3% agarose gel containing ethidium bromide was used in TAE buffer (Sambrook and Russel, 2001). Gel loading buffer was added to samples (2% of total sample volume). A 1 kb plus ladder (Invitrogen) was used to estimate the size and concentration of DNA fragments.

2.4.4 Isolation of DNA fragments by gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis (2.4.3), and a well was made in the gel by cutting out a piece of the gel right below the DNA band. A dialysis-membrane was inserted into the well to make a barrier for movement of the DNA, and the well was filled with TAE buffer. Electrophoresis was resumed until the DNA fragments had moved into the well. The TAE buffer in the well was then

collected using a pipette, and the DNA fragments purified using phenol extraction and ethanol precipitation.

2.4.5 Ligation

Ligation was performed with T4 DNA ligase (3 u/µl, Promega) according to manufacturer instructions. Before ligation the DNA was heated to 45°C for 5 minutes to break hydrogen bonds. The reaction was set at room temperature for 3 hours. It was used an insert: vector ratio (in moles) of 1.3: 1 for inserts of 0.5-3 kb, and 5: 1 for inserts smaller than 100 bp. Dot spot analysis was used to estimate DNA concentrations. When the olignucleotides (~50 bp) was ligated in to the +19/SK+- vector (~5.2 kb) the ratio was 20 ng: 200 ng. When the chimeric *rbcL*: GUS gene (2.2 kb) was ligated in to the pCrc32-vector (~11 kb) the ratio was 300 ng: 800 ng.

2.4.6 DNA sequencing

2 µg of the constructs p+9 and pM cloned into the plasmid pCrc32 were sequenced by the Sanger dideoxy method (MWGBiotech, Martinsried, Germany) to verify that the mutated sequences were correct. It was used a GUS primer (CGCGCTTTCCCACCAACGCTG).

2.4.7 DNA/ RNA quantification

Estimation of DNA concentrations was done by dot spot analysis (Sambrook & Russell 2001). More accurate measurements of DNA and RNA concentrations were done by spectrophotometer at 260 nm (Sambrook & Russell 2001).

2.5 Bioinformatics

Prediction of RNA secondary structures was done with the MFOLD computer folding program, where prediction of RNA secondary structure is based on calculation of the minimum free energy (Zuker, 2003).

2.6 Hybridization analysis

2.6.1 Probes

For Northern blot and slot-blot a GUS probe was made from the entire 1.9 kb coding fragment of the GUS gene. The probe was made by *BamHI/ SacI* digestion of a pBI1221 plasmid (Clontech, CA, USA) (Jefferson et al, 1986). For Southern blot a 0.7 kb *atpB* probe was prepared from the plasmid pCrc*atpB* (Blowers et al., 1990) by *HpaI* / *EcoRV* restriction digesting. Both probes were radio labelled with [α-32P]-dCTP (Amersham Biosciences, Buckinghamshire, UK) by random primer labelling using the Klenow fragment of DNA polymerase I (Feinberg & Vogelstein, 1983).

2.6.2 DNA slot blot

The DNA slot blot was performed using 0.5 µg DNA isolated from *Chlamydomonas* and denatured at 65 ºC with 0.3 N NAOH. The DNA was transferred to a Zeta-Probe nylon membrane (Bio-Rad) in a slot blot apparatus (PR600, Hoefer Scientific Instruments). The DNA was fixed to the membrane by cross-linking with UV light (254 nm) for 3 min (Blowers at al., 1990). The membrane was hybridized to the radio labelled GUS probe according to protocol (Church & Gilbert, 1984). Washing the membrane of excess probe was carried out according to the Bio-Rad protocol. The pattern of hybridization was visualized on X-ray film (Kodak BioMax MS) by autoradiography. The membrane and the film were incubated at -80ºC in a cassette containing an intensifying screen. The strength of the radioactive signal on the membrane was measured using a Geiger counter before the incubation because the exposure time required depended on the specific activity of the labelled probe and the abundance of the target.

2.6.3 Southern blot

1.5 µg of the DNA isolated from *Chlamydomonas* was *KpnI*/ *HindIII* restriction digested, and separated on a 1% agarose gel next to 1 kb plus ladder. DNA fragments

on the gel were blotted onto a Zeta-Probe nylon membrane (Bio-Rad) according to the Bio-Rad protocol. The membrane was hybridized with radiolabeled *atpB* probe according to protocol (Church & Gilbert, 1984). UV-cross linking, washing and exposure to X-ray film was carried out as in section 2.5.2 (DNA slot blot).

2.6.4 Northern blot

The Northern analysis was performed using 4 µg RNA isolated from *Chlamydomonas* growing in a dark/light regime, cells harvested at the end of the dark period. The RNA was denatured by formaldehyde and separated on a 1.3% agarose/ formaldehyde gel at 60 mA constant current. The RNA on the gel was transferred to a Zeta-Probe nylon membrane (Bio-Rad) according to the Bio-Rad protocol. The membrane was hybridized to the radiolabeled GUS probe according to protocol (Church & Gilbert, 1984). UV-cross linking, washing and exposure to X-ray film was carried out as in section 2.5.2 (DNA slot blot) (Figure 2-7).

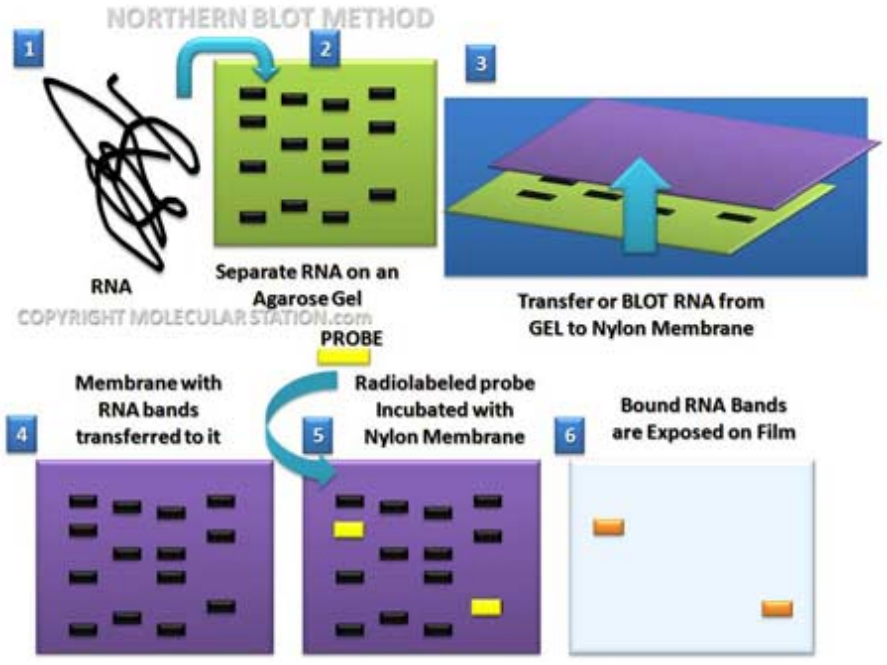


Figure 2-7: Northern blot. *RNA is separated on an agarose gel, blotted on a membrane, radiolabeled and exposed on film. Illustration from http://www.molecularstation.com*

3. Results

3.1 Subcloning

3.1.1 Cloning of oligonucleotides in vector +19/SK+

A first cloning step was performed to induce mutations in the *rbcL* 5' UTRs. The *rbcL* 5' UTRs were located in pBluescript SK+- based plasmids. One of the plasmids (+19/SK+) had a GUS coding region fused 3' to the *rbcL* 5' UTR. The other plasmid (p+93(*EcoRV*)/SK+) did not include the GUS sequence, and the GUS coding region was inserted in an additional cloning step after the mutation of the *rbcL* 5' UTR.

The obtained complementary oligonucleotides were annealed (as described in section 2.4.1). To induce mutations in the *rbcL* 5' UTRs the annealed oligonucleotides +9 and M (figure 2-3) were ligated into plasmid +19/SK+ (figure 2-4) and p+93(*EcoRV*)/SK+ (figure 2-5), respectively (2.4.5).

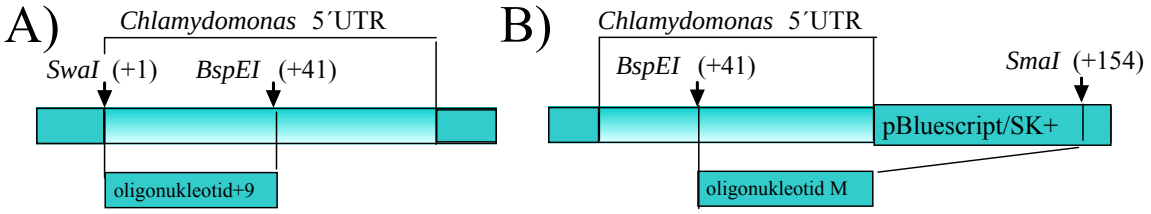


Figure 3-1: The Chlamydomonas rbcL 5'UTRs with the positions of the oligonucleotides +9 and M cloned into the gene. *Positions of restriction sites are relative to rbcl start of transcription. A) Oligonucleotide +9 cloned into the 5'UTR between the restriction sites SwaI (+1) and BspEI (+41). B) Oligonucleotide M cloned into Chlamydomonas 5'UTR between the restriction sites BspEI (+41) and SmaI (+154).*

The double stranded +9-oligonucleotide (50bp) was ligated into *SwaI*/ *BspEI* digested p+19/SK+ (figure 3-1A). This maintained the sequence of the *rbcL* 5'UTR and added 9 nucleotides at the 5'terminus of the transcript. The new vector was named p+9/SK+.

The annealed M-oligonucleotide (50bp) was ligated into *BspEI*/ *SmaI* digested p+93(*EcoRV*)/SK+ (figure 3-1B). This introduced changes in the *rbcL* 5'UTR sequence in positions +49 to +62 (relative to start of transcription). The restriction

digestion of the plasmid also removed a part (61 bp) of the plasmid sequence. The new vector was named pM/SK+.

Transformation was performed with the ligated vectors into competent *E.coli* cells. (2.1.2). The transformants were selected on the basis of ampicillin resistance.

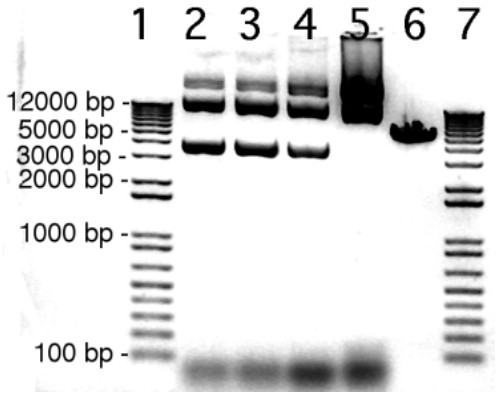
3.1.2 Verifying the ligation

Transformed *E.coli* cells were grown in 3 ml liquid cultures, and plasmids were isolated by mini-prep (2.1.3). To verify the ligation, the isolated plasmids were cut with various restriction enzymes (2.4.2) and run on agarose gels (2.4.3).

To verify the cloning, isolates from p+9/SK+ and pM/SK+ were digested with *SwaI* and *SmaI*, respectably. The successfully mutated plasmids should have lost a restriction site upon the ligation of the oligonucleotides into the vector. *SwaI* digested p+9/SK and *SmaI* digested pM/SK+ should still be uncut and circular if the inserts had been present into the plasmid. In an agarose gel the mutated plasmids should appear as supercoiled DNA.

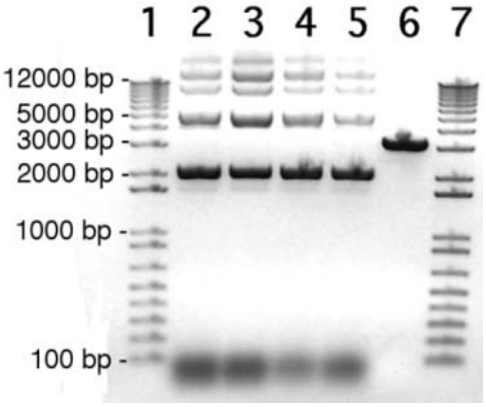
As seen in the photos of the gel (Figure 3-2A) mini-prep isolate nr. 1-3 containing DNA from p+9/SK+ transformants lacked the *SwaI* restriction site. Isolate nr. 4 did not appear as expected in the gel, and was excluded from further testing. All isolates obtained from pM/SK+ transformants contained plasmids lacking the *SmaI* restriction site (Figure 3-2B). Only transformant nr.3 was chosen for further testing.

A)



1: 1 kb plus DNA ladder
2-5: p+9/SK+ uncut by SwaI
6: control: p+19/SK+ cut by SwaI
7: 1 kb plus DNA ladder

B)

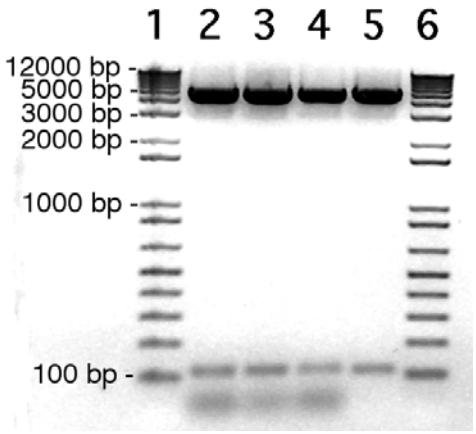


1: 1 kb plus DNA ladder
2-5: pM/SK+ uncut by SmaI
6: control: p+94(EcoRV)/SK+ cut by SmaI
7: 1 kb plus DNA ladder

Figure 3-2: Control of the cloning of +9 into p+19/SK+ and M into p+19(EcoRV)/SK+. *A) SwaI digested p+9/SK+ isolated from transformed E.coli. B) SmaI digested pM/SK+ isolated from transformed E.coli.*

To additionally check for the presence of the modified sequences in the plasmids two different restriction digests were used.

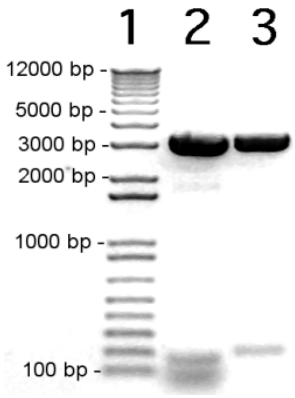
Plasmid p+9/SK+ from transformants nr 1-3 were *BspEI*/ *XhoI* digested. A mutated plasmid should release a small fragment of 124 bp, while an original +19/SK+ vector should release a fragment of only 115 bp. The difference in number of base pairs is caused by the inserted 9-nucleotide addition. As seen in figure 3-3 all p+9/SK+ vectors were cut twice and released a small fragment of ~120 bp.



1: 1 kb plus DNA ladder
2-4: p+9/SK+ cut by BspEI- XhoI
5: control: p+19/SK+ cut by BspEI- XhoI
6: 1 kb plus DNA ladder

Figure 3-3: Control of the cloning of +9 into p+19/SK+. *Checking the size of the BspEI-XhoI fragments in plasmid DNA isolated from p+9/SK+ transformants. The larger fragments are ~5 kb and the smaller fragments are ~0.1 kb.*

Plasmid pM/SK+ from transformant nr 3 was *XbaI*/ *XhoI* digested. A modified plasmid should release a small fragment of 168 bp. An original p+93(*EcoRV*)/SK+ vector should release a fragment of 229 bp. The fragments differ in size because the insertion of the M-oligonucleotide leads to a sequence deletion in the original vector (figure 3-4B). As seen in figure 3-4 the isolated pM/SK+ was cut twice and released a small fragment of expected size.



1: 1 kb plus DNA ladder
2: pM/SK+ (from transformant nr.3) cut by XhoI-XbaI
3: control: p+94(EcoRV)/SK+ cut by XhoI-XbaI

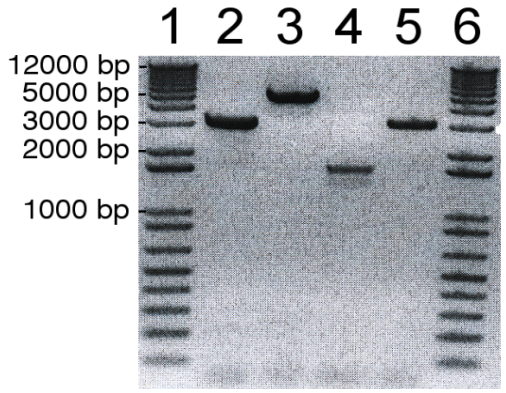
Figure 3-4: Control of the cloning of M into p+93(EcoRV)/SK+. *Checking the size of the XhoI-XbaI fragments in plasmid DNA isolated from pM/SK+ transformants. The larger fragments are ~3 kb and the smaller fragments are ~0.2 kb.*

The larger fragment resulting from the *XbaI*/ *XhoI* digestion was expected to be ~5 kb, but it was only ~3 kb in both pM/SK+ and in the control (figure 3-4). This was because the ~2 kb GUS coding sequence was not included in the original

p+93(*EcoRV*)/SK+ vector. To complete the reporter construct it was therefore necessary to ligate the missing GUS sequence into the pM/SK+ vector.

3.1.3 Introduction of GUS into vector pM/SK+

+19/SK+ was used as a source of the GUS gene. The required 1.9 kb GUS sequence was released from the vector by *BamHI/ XbaI* restriction digestion. The pM/SK+ vector was prepared for ligation by restriction cutting with the same restriction enzymes. The GUS coding sequence (~2 kb) and the restriction cut pM/SK+ vector (~3.2 kb) were isolated by gel electrophoresis (2.4.4). Sample concentration of the two DNA fragments were determined by dot spot analysis (2.4.7), and ligation was performed (2.4.5). To amplify the vector it was transformed into competent *E.coli* cells (2.1.2), grown in cultures (2.1.1), and isolated with min-prep (2.1.3). To verify the size of the new pM/SK+ vector it was restriction digested with *XbaI* to make it linear, and run on an agarose gel (2.4.3).



1: 1 kb plus DNA ladder
2-5: Isolate nr 1-4 cut by XbaI
1: 1 kb plus DNA ladder

Figure 3-5: Control of GUS into pM/SK+. *Checking the size of the XbaI digested plasmids isolated from pM/SK+ transformants. Isolate nr 2 contained the ~5 kb vector.*

Only isolate nr 2 contained the 5.2 kb vector (figure 3-5). Isolates nr 1 and 4 contained the pM/SK+ vector religated without the GUS insert and isolate nr. 3 contained a ~1.6 kb plasmid of unknown origin. Transformant nr 2 was used for further analyses.

3.1.4 Cloning of *rbcL* 5'UTR: GUS into vector pCrc32

A second cloning step was performed to insert the mutated [*rbcL* 5'end: GUS coding region] constructs into an ~11 kb transformation vectors. They were inserted 5' to *psaB* 3'UTRs in order to complete the gene constructs.

Maxi-prep (2.1.3) was performed on *E.coli* cultures transformed with successfully mutated plasmids. *XbaI/ XhoI* digestion of the isolates yielded in two DNA fragments of 2.2 bp and 3.0 bp. The 2.2 kb fragment contained the mutated *rbcL*: GUS construct, and was isolated by gel electrophoresis (2.4.4). The transformation vector pCrc32 (~11kb) was also *XbaI*/ *XhoI* digested, and the larger DNA fragment, of ~9 kb, was isolated by gel electrophoresis.

The *rbcL* 5'end: GUS constructs were ligated into the restriction-digested pCrc32 vector (Figure 3-6). The cloned pCrc32 vectors containing p+9/SK+ and pM/SK+ were named pCrc32/+9 and pCrc32/M, respectively.

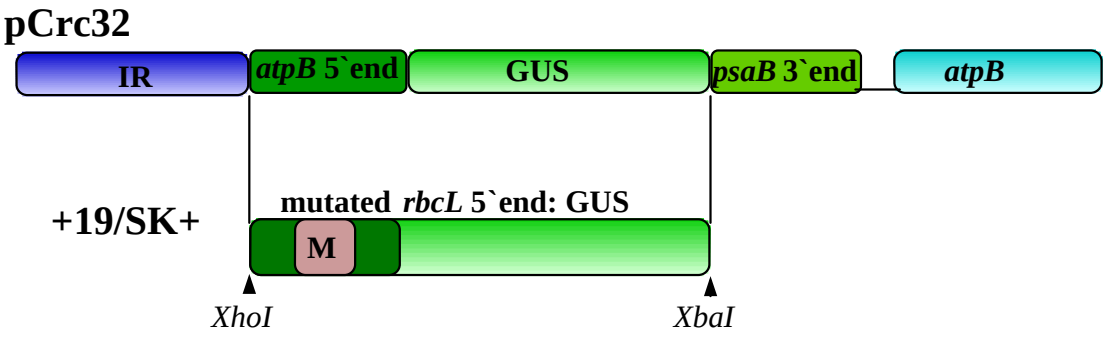


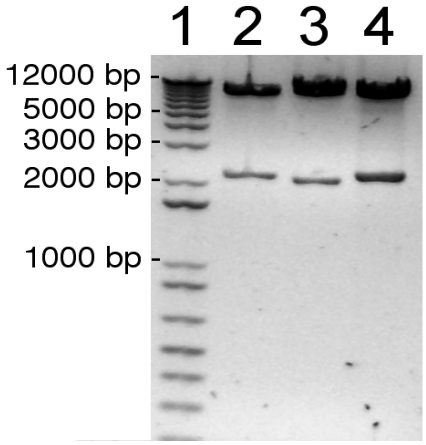
Figure 3-6: The rbcL 5'UTR: GUS construct ligated into vector pCrc32. *The original atpB 5'end and the coding region of GUS in the pCrc32 are exchanged with the mutated rbcL: GUS construct. M indicates the mutated sequence in the rbcL 5'UTR.*

In order to amplify pCrc32/+9 and pCrc32/M, transformation was performed with the ligated vectors into competent *E.coli* cells (2.1.2). Transformants were selected on the basis of ampicillin resistance.

3.1.5 Verifying the cloning

To verify the cloning, the transformed *E.coli* cells were grown in 3 ml liquid cultures, and plasmids were isolated by min-prep (2.1.3). The isolated plasmids were restriction digested (2.4.2) and separated on agarose gels (2.4.3).

Both pCrc32/+9 and pCrc32/M were *XhoI*/ *XbaI* digested for size confirmation. The cloned vectors appeared in the gel as two linear fragments; one of ~8.8 kb and one of ~2.2 kb (*rbcL*: GUS) (Figure 3-7). This was a verification of that there were GUS constructs in the vectors.



1: 1 kb plus DNA ladder
2: pCrc32+9/SK+ cut by XhoI-XbaI
3: pCrc32M/SK+ cut by XhoI-XbaI
4: control, original pCrc32 cut by XhoI- XbaI

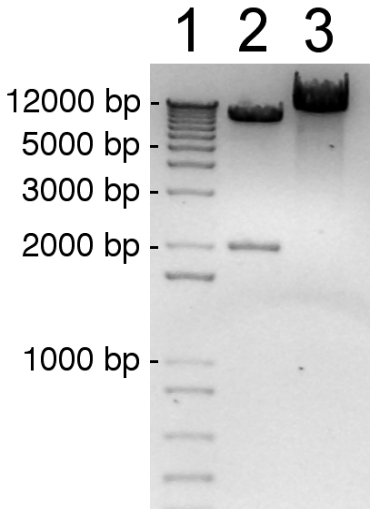
Figure 3-7: Control of the cloning of p+9/SK+ and pM/SK+ into pCrc32. *Checking the size of the XhoI-XbaI fragment in plasmid DNA isolated from pCrc+9/SK+ and pCrcM/SK+ transformants. The larger fragments are ~9 kb and the smaller fragments were ~2 kb.*

A second test was performed on each of the plasmids to verify that the *atpB*: GUS construct of the original pCrc32 vector has been replaced by the *rbcL*: GUS construct.

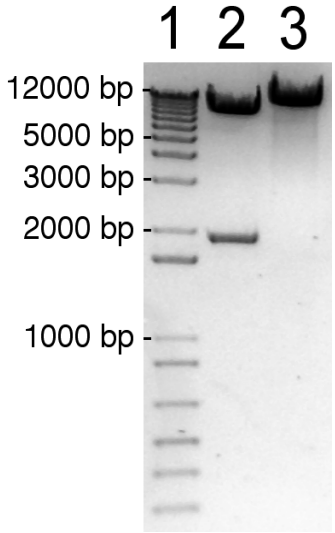
BspEI was used to digest pCrc32/+9. There should be two restriction sites in the cloned transformation vector. One in the inserted *rbcL* sequence and one outside the inserted fragment in the *Chlamydomonas* sequence.

SwaI was used to digest pCrc32/M. The clone should contain two *SwaI* sites. There is one *SwaI* site in the mutated insertion and one in the original transformation vector.

The cloned vectors appeared in the gel as two fragments (Figure 3-8A and B). This was a verification of the presence of *rbcL*: GUS constructs in the vectors.



A)
Checking for p+9-insert by *BspEI* digestion of pCrc+9/SK+ transformants
1: 1 kb plus DNA Ladder
2: pCrc32+9/SK+ cut by BspEI
3: control, original pCrc32 cut by BspEI



B)
Checking for pM-insert by *SwaI* digestion of pCrcM/SK+ transformants
1: 1 kb plus DNA Ladder
2: pCrc32+M/SK+ cut by SwaI
3: control, original pCrc32 cut by SwaI

Figure 3-8: Control of the cloning of p+9/SK+ and pM/SK+ into pCrc32*. A) BspEI digested pCrc+9/SK+. B) SwaI digested pCrcM/SK+. The cloned vectors appeared in the gel as two fragments. The controls were only cut once.*

3.1.6 Sequencing

After verification of cloning by restriction cutting, the final transformation vectors were sent to MWGBiotech, Martinsried, Germany for sequencing (2.4.6). The analysed sequence included the *rbcL* 5'end sequence and some of the GUS gene. This confirmed that the sequence in pCrc32/M was as expected. But the sequence in pCrc32/+9 was not. The first nucleotide of the original *rbcL* 5'UTR was missing. This probably happened during ligation of the +9-oligonucleotide into the *SwaI/*

BspEI sites of p+19/ SK+. The deletion would lead to a change in the secondary structure of the 10-nucleotide *cis*-acting structure. This is already known through earlier analyses to render the transcript unstable. It was decided to continue testing the construct anyway.

3.1.7 Transformation of *Chlamydomonas*

Maxi-prep (2.1.3) was performed on cultures with *E.coli* cells transformed with successfully mutated plasmids. The plasmids pCrc32/+9 and pCrc32/M were introduced into the chloroplast of *Chlamydomonas* CC-373 (2.2.1) by micro projectile bombardment (2.2.4). This allowed for recombination to occur between the homologous sequences in the chloroplast DNA and the plasmids (Figure 3-9). Transformants (restored *atpB* gene, not light-sensitive) were selected by exposing them to bright light.

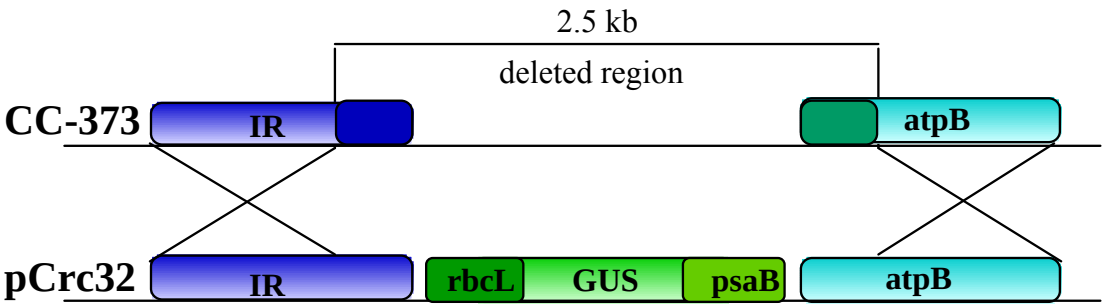


Figure 3-9: Recombination between the homologous sequences in the chloroplast DNA and the plasmids. *The chimeric rbcL 5'end: GUS: psaB 3'end is introduced into the CC-373 through homologous recombination. The crosses indicate the recombination event.*

3.2 Screening for GUS gene transformants by Slot blot.

The amount of the chimeric *rbcL*: GUS: *psaB* genes in the transformants were determined by performing a Slot blot (2.6.2) on total genomic DNA using a GUS probe (2.6.1) (Figure 3-10). This was done with DNA isolated from six randomly selected transformants. Isolates from transformants with the highest GUS content gave the strongest autoradiogram signal, and were used for further analyses. For construct p+9 transformants number 2 and 6 were chosen (Figure 3-10A). For construct pM transformants number 2, 3, 4 and 6 were chosen (Figure 3-10B).

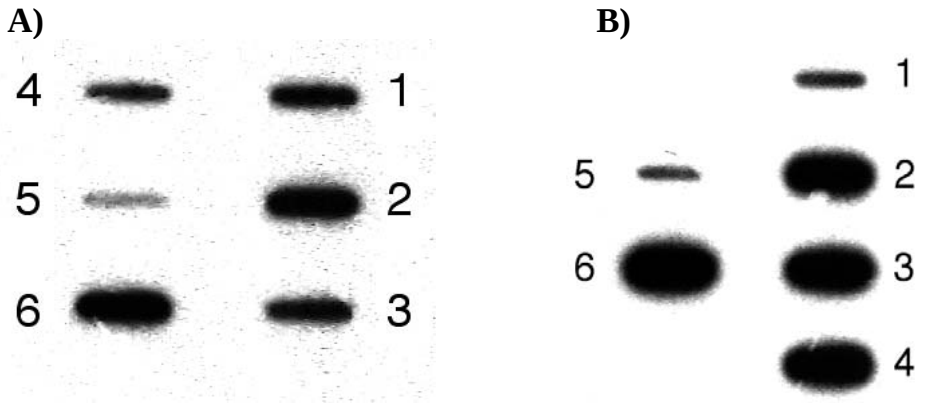


Figure 3-10: Autoradiogram showing amount of the chimeric GUS gene in the transformants. *A) 6 isolates from different p+9-transformants. Isolates number 2 and 6 gave the strongest signals. B) 6 isolates from different pM-transformants. Isolates number 2, 3, 4 and 6 gave the strongest signals.*

3.3 Determination of homoplasmicity (Southern analysis)

Southern analysis was performed on the isolated DNA to determine the homoplasmicity percentage of the selected transformants (2.6.3). There are 50-80 copies of the DNA chromosome in the chloroplast of a *Chlamydomonas* cell. Each of these chromosomes can be transformed with the mutated plasmid. The homoplasmicity percentage reflects the number of chromosomes that are transformed with the new sequence.

DNA isolated from the transformants was cut with restriction enzymes *KpnI* and *HindIII*. If the chloroplast genome had incorporated the chimeric *rbcL: GUS: psaB* construct, this digestion would release a fragment of ~5.5 kb. A chloroplast genome without the chimeric GUS gene insert would release a fragment of only ~3 kb.

The digested fragments were separated on an agarose gel and visualized through a Southern blot using an *atpB* probe (2.6.1). Isolated total DNA appears as "a smear" in the gel if it is restriction digested (Figure 3-11A and 3-12A). The position of the DNA is shown relative to the 1 kb Plus DNA ladder and a ruler. The ruler was later used to estimate the sizes of the *atpB*-fragments shown in the autoradiogram.

The autoradiogram of the Sothern blot is shown in figure 3-11B and 3-12B. For further analysis, the transformants with highest homoplasmicity were used. For construct p+9 transformants the digested isolates had barely visible 3 kb fragments, both chloroplasts were estimated to have a homoplasmicity of ~90%, and were used for further analysis. For construct pM transformants the transformant number 6 had a very weak 3 kb signal, was also estimated to have a homoplasmicity of ~90% and was used for further analysis.

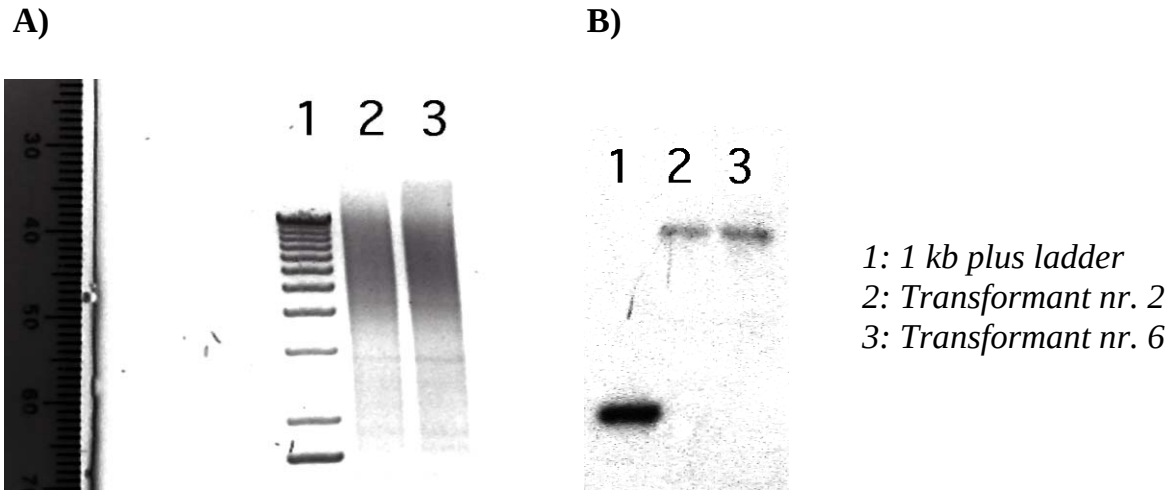


Figure 3-11: The degree of homoplasmicity of the p+9 transformants. *A) Picture of the gel showing the presence of restriction cut DNA. A ruler and the 1 kb plus ladder indicate positions of the expected fragments. B) Autoradiogram showing the restriction cut fragments. The ~3 kb fragments are barely visible and the homoplasmicity of both transformants were estimated to ~90%.*

A) **B)**

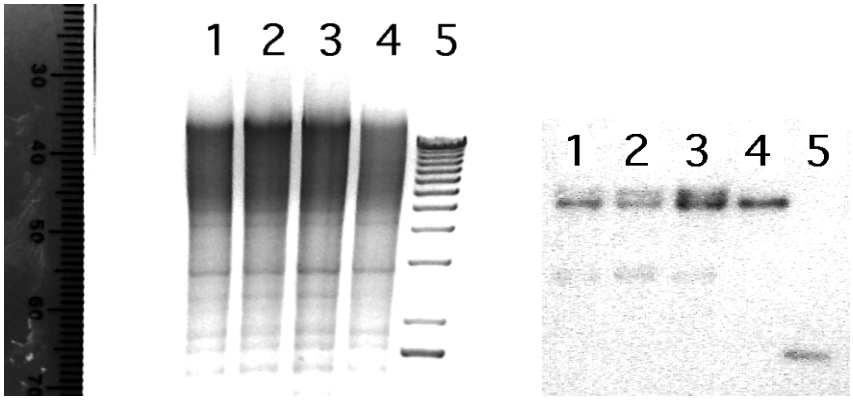


Figure 3-12: The degree of homoplasmicity of the pM transformants. *A) Picture of the gel showing the presence of restriction cut DNA. A ruler and the 1 kb plus ladder indicate positions of the expected fragments. B) Autoradiogram showing the restriction cut fragments. The transformant number 6 ~3 kb fragment is barely visible and the homoplasmicity was estimated to ~90%.*

3.4 Determination of GUS transcript accumulation (northern analysis)

To investigate the stability of the *rbcL*: GUS: *psaB* transcripts, transcript levels were detected by northern hybridization (2.6.4). A stable transcript would lead to accumulation of GUS RNA in the chloroplast. This would be detected in a Northern blot hybridized with a GUS probe (2.6.1). Highly unstable transcripts would not reach detectable levels.

Isolated RNA (2.2.7) from the transformants were separated on agarose gels (Figure 3-13A and 3-14A) and transferred to Zeta-Probe membranes. The membranes were hybridized to the GUS probes and exposed to X-ray films. The autoradiograms showed the accumulation of the chimeric GUS transcripts (Figure 3-13B and 3-14B). RNA concentrations were reflected in the strength of the signals.

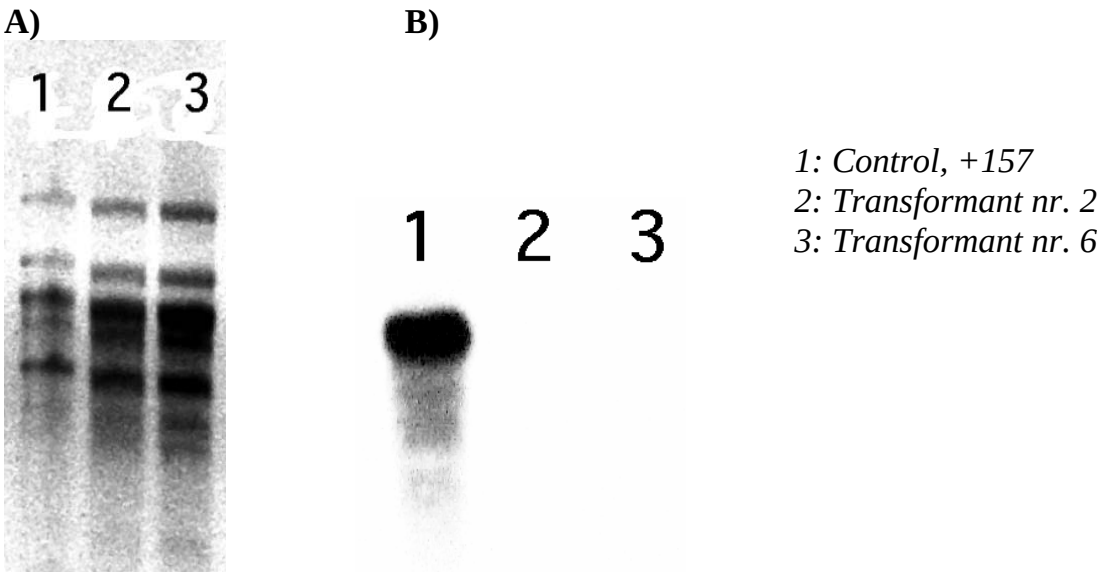


Figure 3-13: RNA isolated from the p+9 transformants. *A) Agarose gel with total genomic RNA. Showing the presence of RNA. B) Autoradiogram showing abundance of chimeric GUS transcripts in transformants. Only control-transcripts are visible. No accumulation of GUS transcripts from p+9 transformants.*

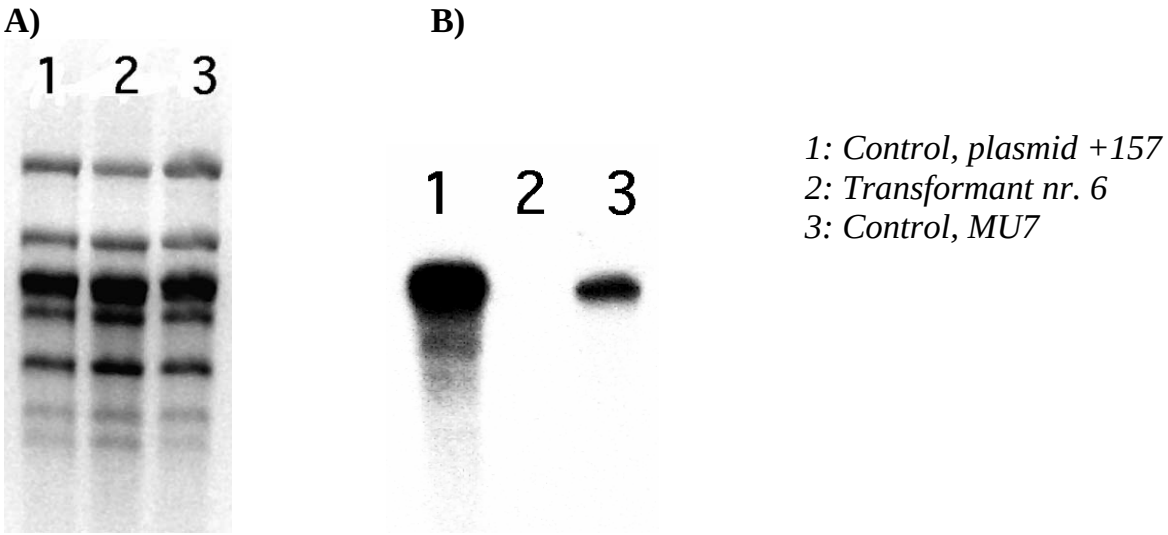


Figure 3-14: RNA isolated from the pM transformants. *A) Agarose gel with total genomic RNA. Showing the presence of RNA. B) Autoradiogram showing abundance of chimeric GUS transcripts in transformants. Only the 100% and the 10% controls visible. No accumulation of GUS transcripts in pM transformants.*

RNA isolated from a transformant named 157+ was used as control for both transformants. The isolate has a 100% GUS RNA level relative to dark-isolated mRNA levels in a transformant harbouring a GUS gene with an unmodified *rbcL* 5'end. This would have been the expected GUS transcript level in a p+9 transformant if the induced modifications had not affected the stability of the transcript.

For the pM transformant an additional control (MU7) with a 10% RNA level was used. This was because the *rbcL* enhancer sequence was not included in the pM-gene construct. The chimeric gene would therefore be transcribed to a ~10% mRNA level compared to an equivalent chimeric gene containing the enhancer.

Only controls were seen in the autoradiograms. Accumulation of the p+9 and pM GUS transcripts were not detectable by northern blot assay.

CONCLUSION

The p+9-construct

A 5’ addition of 9 nucleotides to the 5’UTR of the *Chlamydomonas rbcL* gene and a deletion of the first nucleotide of the original transcript made the chimeric *rbcL* 5’end: GUS transcripts highly unstable. The homoplasmicities of the transformants were high, estimated to about 90% by Southern analysis. The transcript levels in the transformed *Chlamydomonas* chloroplasts were not detectable by northern blot.

From this it can be concluded that the induced modifications significantly destabilized the transcripts. However because two different modifications were present in the construct which both, independently, could abolish transcript accumulation, it cannot be decided whether both modifications, or just one of the induced alterations destabilized the GUS transcripts.

The pM-construct

Modifications in the sequence of the second stem-loop of the 5’UTR of the *Chlamydomonas rbcL* transcripts (positions +50 to +63 relative to start of transcription) also led to highly unstable GUS transcripts in the cells. The homoplasmicity of the transformant was estimated to ~ 90% by Southern analysis. Transcript accumulation was not detectable by northern analysis. This indicates that there are nucleotide(s) within this changed area essential for transcript stability.

4. Discussion

In this study two different reporter genes were constructed to analyze how the 5'UTR of the *rbcL* mRNA affects the stability of the transcript. Two different modifications were introduced at *rbcL* 5'UTRs. The 5'UTRs were fused to GUS reporter genes and the chimeric genes were cloned into *Chlamydomonas* chloroplast genomes in order to investigate the *in vivo* effect of the modifications. One modification added 9 nucleotides to the 5' terminus of the transcript and deleted the first nucleotide of the original transcript. The other changed the nucleotides in a 5'UTR stem-loop. It was found that both *rbcL* 5'UTR modifications destabilize the GUS transcripts and significantly reduses transcript accumulation.

4.1 The p+9 construct's effect on transcript stability

The p+9-construct's effect on transcript stability

The addition of 9 nucleotides to the 5'terminus of the *rbcL* transcript (Figure 4-1) was introduced to determine whether this addition would destabilize the transcript. However, during ligation there was also made a single-nucleotide deletion in the construct affecting the secondary structure of the +38 to +47 *cis*-acting sequence.

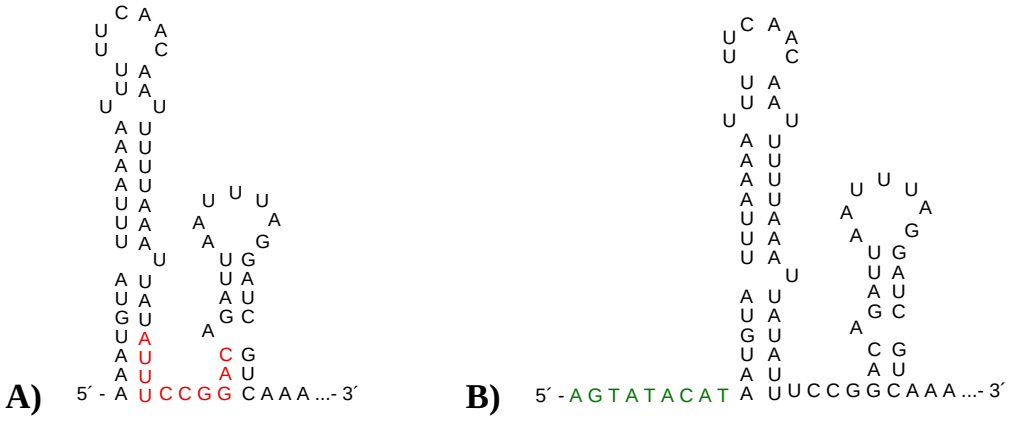


Figure 4-1: Predicted rbcL 5'UTR RNA secondary structures. *A) Unmodified sequence of the rbcL 5'UTR with sequence +38 to +47 shown in red. B) The chloroplast rbcL 5'UTR of Chlamydomonas with 9 additional nucleotides (shown in green) and a deleted +1 nucleotide (resulting in a longer ss-sequence between the loops).*

Changes in the secondary structure of the 10-nt *cis*-acting sequence are already known to render the transcript unstable. Therefore the transcript destabilization observed in this analysis could be a result of both the inserted modifications or only one of them. As a result of this no definitive conclusion about the effect of the added 9 nucleotides can be drawn.

Previous work affecting the 5' terminal sequence

Previous experiments have shown that adding up to 8 nucleotides to the 5' terminus does not significantly affect transcript stability. 10 or more additional nucleotides abolish transcript accumulation (Klein, U., personal communication).

The destabilizing effect of 10 or more additional nucleotides at the 5'terminus might be because the introduced nucleotides interfere with the binding of a stabilizing *trans*-acting factor. It is also possible that the additional nucleotides provide binding sites for 5'-3' RNases.

When less nucleotides are added, the introduced 5' sequence might not be long enough to bind RNases or to interfere with the binding of a *trans*-acting factors. It has not yet been investigated how 5'end processing affects the mutated mRNAs. As adding 8 nucleotides or less does not significantly change transcript stability it is possible the added nucleotides are removed by 5'end processing, resulting in WT-*rbcL* 5'ends in the mature mRNA.

4.2 The pM construct's effect on transcript stability

The pM-construct's effect on transcript stability

The findings presented in this thesis show that when it is introduced changes in the upper part of the small 5'UTR stem-loop (position +50 to +62 relative to start of transcription) (figure 4-2) that does not disturb the secondary structure, the transcripts are destabilized. This means that there are one or several nucleotides in this area important for transcript stability.

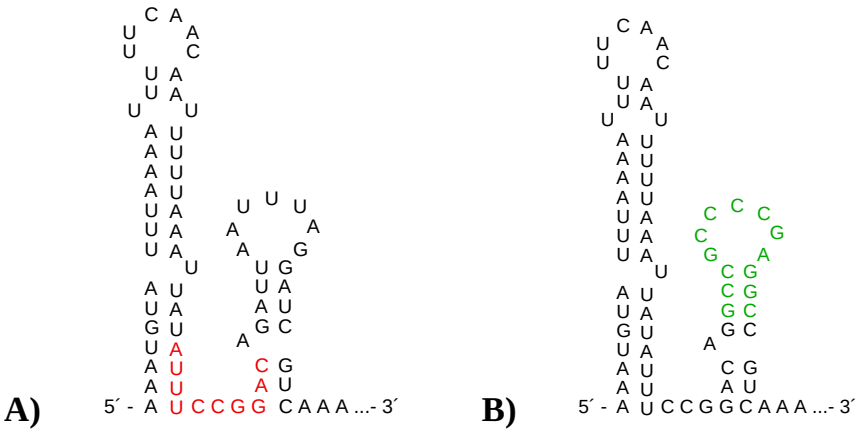


Figure 4-2: Predicted rbcL 5'UTR RNA secondary structures. *A) Unmodified sequence of the rbcL 5'UTR with sequence +38 to +47 shown in red. B) The chloroplast rbcL 5'UTR of Chlamydomonas with the changed sequence in positions +50 to +62 shown in green.*

The proximity of this newly found stabilizing nucleotide(s) to the previously found +38 to +47 element makes it likely that they are part of the same *cis*-acting sequence. Stabilizing elements in the 5'UTR of chloroplast transcripts are believed to be *cis*-acting sites recruiting sequence-specific *trans*-factors (Suay et al., 2005).

The role of the nucleotides located in the second stem-loop might be providing a binding site to the postulated *trans*-acting factor. As the secondary structure of the stem-loop was not disturbed, the stabilizing nucleotide(s) are likely to have a sequence specific interaction with the postulated *trans*-acting factor.

Previous work on the second stem loop

Previously there has been done a deletion of the +56 to +63 sequence which did not have a significant effect on transcript stability (Salvador et al., 2004b). The deletion disrupted the secondary structure of the second stem loop from position +50 to +55, but maintained the nucleotides at positions +50 to +53 in a loop-structure (figure 4-3).

There have also been inserted point mutations in nucleotides +48, +49, +53 and +59, which also did not have a significant effect on transcript stability (Anthonisen et al., 2001) (figure 4-3). The Northern blot membrane containing hybridized +49 mutated RNA presented by Anthonisen et al. (2001) reveals a somewhat weaker signal than

the control (~50%), implying that also this nucleotide can have an impact on transcript stability. Alternatively, this could be a result of lower homoplasmicity.

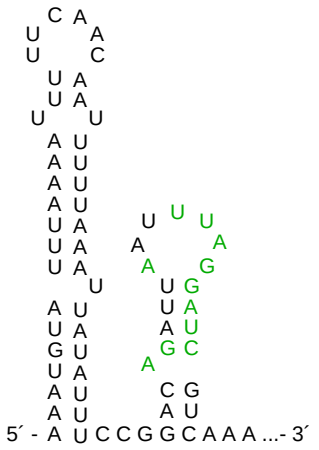


Figure 4-3: Previous work on the second stem loop. *In the second stem-loop nucleotides previously changed or deleted without effecting the transcript stability are shown in green.*

The location of nucleotide(s) important for transcript stability

In light of these results, it seems that nucleotide(s) important for transcript stability can be found between nucleotides +50 and +55, not including nucleotide +53. This because the deletion of the + 56 to +63 sequence and the point mutation in +53 did not have a significant effect on transcript stability. The +49 nucleotide might also be of importance (figure 4-4).

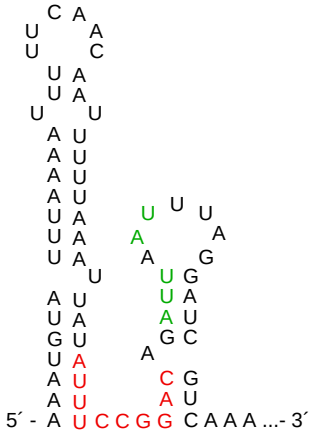


Figure 4-3: Location of nucleotide(s) important for transcript stability. *The location of the nucleotide(s) in the second stem loop that might be of importance for transcript stability are shown in green. The location of the previously found cis-acting element is shown in red.*

It is not known whether the loop-configuration in nucleotides +53 to +55 is of importance as both the pM-construct and the deletion performed by Salvador et al. (2004) preserved this configuration.

It is also open whether all the nucleotides +50, +51, +52, +54 and +55 are necessary for transcript stability, or if a deletion/ change in one or some of these nucleotides will destabilize the transcript. It is possible that several of these nucleotides are necessary, but that this part of the *cis*-acting element allows for small changes, as point mutations, without disturbing or losing the stabilizing effect. The stability in the +53 mutant and the reduced stability in the +49 mutant could be a result of this.

Increased number of hydrogen bonds

In the WT-*rbcL* 5'UTR the second stem loop consists primarily of adenine and thymine. The mutation made the sequence guanine/ cytosine-rich with an increased number of hydrogen bonds. This did not affect the stem-loop secondary structure, but might influence the stability of the transcript by enhancing the rigidity of this area, which may change the nature of the interaction with a *trans*-acting factor.

FUTURE PERSPECTIVES

Future mutation analyses of the *Chlamydomonas rbcL* 5'UTR effect on transcript stability should determine the location of nucleotide(s) important for transcript stability in the small stem-loop of the 5'UTR. To determine whether an addition of 9 nucleotides to the 5'terminus of the *rbcL* transcript has a destabilizing effect it should be made a new mutation construct, without any additional changes.

Finding proteins binding to the *rbcL* 5'UTR can also give information on how the 5'UTR affects the longevity of *Chlamydomonas rbcL* transcripts.

Bibliography

ALDRICH, J., CHERNEY, B., MERLIN, E., WILLIAMS, C. & METS, L. (1985). Recombination within the inverted repeat sequences of the *Chlamydomonas reinhardii* chloroplast genome produces 2 orientation isomers. *Curr Genet* **9,** 233-238.

ALLISON, L. A. (2000). The role of sigma factors in plastid transcription. *Biochimie* **82,** 537-548.

ANTHONISEN, I., KASAI, S., KATO, K., SALVADOR, M. & KLEIN, U. (2002). Structural and functional characterization of a transcription-enhancing sequence element in the *rbcL* gene of the *Chlamydomonas* chloroplast genome. *Curr Genet* **41,** 349-356.

ANTHONISEN, I. L., SALVADOR, M. L. & 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,** 1024-1033.

ASAYAMA, M., SUZUKI, H., SATO, A., AIDA, T., TANAKA, K., TAKAHASHI, H. & SHIRAI, M. (1996). The *rpoD1* gene product is a principal sigma factor of RNA polymerase in Microcystis aeruginosa K-81. *J Biochem* **120,** 752-758.

BARBROOK, A. C., HOWE, C. J. & PURTON, S. (2006). Why are plastid genomes retained in non-photosynthetic organisms? *Trends Plant Sci* **11,** 101-108.

BARKAN, A. & GOLDSCHMIDT-CLERMONT, M. (2000). Participation of nuclear genes in chloroplast gene expression. *Biochimie* **82,** 559-572.

BAUMGARTNER, B. J., RAPP, J. C. & MULLET, J. E. (1993). Plastid genes encoding the transcription translation apparatus are differentially transcribed early in barley (Hordeum-Vulgare) chloroplast development - evidence for selective stabilization of *PSBA* messenger-RNA. *Plant Physiol* **101,** 781-791.

BLOWERS, A. D., BOGORAD, L., SHARK, K. B. & SANFORD, J. C. (1989). Studies on *Chlamydomonas* chloroplast transformation: Foreign DNA can be stably maintained in the chromosome. *Plant Cell* **1,** 123-132.

BLOWERS, A. D., ELLMORE, G. S., KLEIN, U. & BOGORAD, L. (1990). Transcriptional analysis of endogenous and foreign genes in chloroplast transformants of *Chlamydomonas*. *Plant Cell* **2,** 1059-1070.

BLOWERS, A. D., KLEIN, U., ELLMORE, G. S. & BOGORAD, L. (1993). Functional in vivo analyses of the 3' flanking sequences of the *Chlamydomonas* chloroplast *rbcL* and *psaB* genes. *Mol Gen Genet* **238,** 339-349.

BOCK, R. & TIMMIS, J. N. (2008). Reconstructing evolution: gene transfer from plastids to the nucleus. *BioEssays* **30,** 556-566.

BOHNE, A.-V., IRIHIMOVITCH, V., WEIHE, A. & STERN, D. B. (2006). *Chlamydomonas reinhardtii* encodes a single sigma 70-like factor which likely functions in chloroplast transcription. *Curr Genet* **49,** 333-340.

BOLLENBACH, T. J., SCHUSTER, G. & STERN, D. B. (2004). Cooperation of endo- and exoribonucleases in chloroplast mRNA turnover. *Prog Nucleic Acid Res* **78,** 305-337.

BOLLENBACH, T. J., TATMAN, D. A. & STERN, D. B. (2003). CSP41a, a multifunctional RNA-binding protein, initiates mRNA turnover in tobacco chloroplasts. *Plant J* **36,** 842-852.

BOUDREAU, E., NICKELSEN, J., LEMAIRE, S. D., OSSENBUHL, F. & ROCHAIX, J. D. (2000). The *Nac2* gene of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. *EMBO J* **19,** 3366-3376.

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

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

CARPOUSIS, A. J., MUDD, E. A. & KRISCH, H. M. (1989). Transcription and messenger-RNA processing upstream of bacteriophage-T4. *Mol Gen Genet* **219,** 39-48.

CARTER, M. L., SMITH, A. C., KOBAYASHI, H., PURTON, S. & HERRIN, D. L. (2004). Structure, circadian regulation and bioinformatic analysis of the unique sigma factor gene in *Chlamydomonas reinhardtii*. *Photosynth Res* **82,** 339-349.

CAVALIER-SMITH, T. (2000). Membrane heredity and early chloroplast evolution. *Trends Plant Sci* **5,** 174-182.

CECH, T. R. (1990). Self-splicing of group I introns. *Annu Rev Biochem* **59,** 543-568.

CHURCH, G. M. & GILBERT, W. (1984). Genomic Sequencing. *P Natl Acad Sci USA* **81,** 1991-1995.

DESHPANDE, N. N., BAO, Y. J. & 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. & STERN, D. B. (1998). In vivo evidence for 5' to 3' exoribonuclease degradation of an unstable chloroplast mRNA. *Plant J* **13,** 85-96.

DRAGER, R. G., HIGGS, D. C., KINDLE, K. L. & STERN, D. B. (1999). 5' to 3' exoribonucleolytic activity is a normal component of chloroplast mRNA decay pathways. *Plant J* **19,** 521-531.

DRAGER, R. G., ZEIDLER, M., SIMPSON, C. L. & STERN, D. B. (1996). A chloroplast transcript lacking the 3´ inverted repeat is degraded by 3´to 5´ exoribonuclease activity. *RNA* **2,** 652-663.

DRAPIER, D., SUZUKI, H., LEVY, H., RIMBAULT, B., KINDLE, K. L., STERN, D. B. & WOLLMAN, F.-A. (1998). The chloroplast *atpA* gene cluster in *Chlamydomonas reinhardtii* . Functional analysis of a polycistronic transcription unit. *Plant Physiol* **117,** 629-641.

ESPOSITO, D., HICKS, A. J. & STERN, D. B. (2001). A role for initiation codon context in chloroplast translation. *Plant Cell* **13,** 2373-2384.

FEINBERG, A. P. & VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132,** 6-13.

FREYER, R., KIEFER-MEYER, M.-C. & KOSSEL, H. (1997). Occurrence of plastid RNA editing in all major lineages of land plants. *P Natl Acad Sci USA* **94,** 6285-6290.

GRUISSEM W., ELSNER-MENZEL, C., LATSHAW, S., NARITA, J. O., SCHAFFER, M. A. & ZURAWSKI, G. (1986). A subpopulation of spinach chloroplast tRNA genes does not require upstream promoter elements for transcription. *Nucleic Acids Res* **14,** 7541-7556.

HAJDUKIEWICZ, P. T. J., ALLISON, L. A. & MALIGA, P. (1997). The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *EMBO J* **16,** 4041-4048.

HARRIS, E. H. (2001). *Chlamydomonas* as a model organism. *Ann Rev Plant Phys* **52,** 363-406.

HAYASHI, K., SHIINA, T., ISHII, N., IWAI, K., ISHIZAKI, Y., MORIKAWA, K. & TOYOSHIMA, Y. (2003). A role of the -35 element in the initiation of transcription at *psbA* promoter in tobacco plastids. *Plant Cell Physiol* **44,** 334-341.

HERRIN, D. L. & NICKELSEN, J. (2004). Chloroplast RNA processing and stability. *Photosynth Res* **82,** 301-314.

HESS, W. R. & BORNER, T. (1999). Organellar RNA polymerases of higher plants. *Int Rev Cytol* **190,** 1-59.

HIGGS, D. C., SHAPIRO, R. S., KINDLE, K. L. & 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,** 8479-8491.

HOFFMEISTER, M. & MARTIN, W. (2003). Interspecific evolution: microbial symbiosis, endosymbiosis and gene transfer. *Environ Microbiol* **5,** 641-649.

IGLOI, G. L. & KÖSSEL, H. (1992). The transcriptional apparatus of chloroplasts. *Crit Rev Plant Sci* **10,** 525-558.

JEFFERSON, R. A., BURGESS, S. M. & HIRSH, D. (1986). Beta-glucuronidase from *Escherichia coli* as a gene-fusion marker. . *Proc. Natl. Acad. Sci. USA* **83,** 8447-8451.

KLAFF, P. (1995). mRNA decay in spinach chloroplasts: *psbA* mRNA degradation is initiated by endonucleolytic cleavages within the coding region. *Nucleic Acids Res* **23,** 4885-4892.

KLEIN, R. R. & MULLET, J. E. (1990). Light-induced transcription of chloroplast genes - *psbA* transcription is differentially enhanced in illuminated barley. *J Biol Chem* **265,** 1895-1902.

KLEIN, U., CAMP, J. D. D. & BOGORAD, L. (1992). Two types of chloroplast gene promoters in *Chlamydomonas reinhardtii*. *P Natl Acad Sci USA* **89,** 3453-3457.

KLEIN, U., SALVADOR, M. L. & BOGORAD, L. (1994). Activity of the *Chlamydomonas* chloroplast *rbcL* gene promoter is enhanced by a remote sequence element. *P Natl Acad Sci USA* **91,** 10819-10823.

KOMINE, Y., KIKIS, E., SCHUSTER, G. & STERN, D. (2002). Evidence for in vivo modulation of chloroplast RNA stability by 3'-UTR homopolymeric tails in *Chlamydomonas reinhardtii*. *P Natl Acad Sci USA* **99,** 4085-4090.

KOMINE, Y., KWONG, L., ANGUERA, M. C., SCHUSTER, G. & STERN, D. B. (2000). Polyadenylation of three classes of chloroplast RNA in *Chlamydomonas reinhadtii*. *RNA* **6,** 598-607.

KUCHKA, M. R., GOLDSCHMIDT-CLERMONT, M., DILLENWIJN, J. & ROCHAIX, J. D. (1989). Mutation at the *Chlamydomonas* nuclear NAC2 locus specifically affects stability of the chloroplast *psbD* transcript encoding polypeptide D2 of PSII. *Cell* **58,** 869-876.

LEVY, H., KINDLE, K. L. & 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,** 35955-35962.

LI, F., HOLLOWAY, S. P., LEE, J. & HERRIN, D. L. (2002). Nuclear genes that promote splicing of group I introns in the chloroplast 23S rRNA and *psbA* genes in *Chlamydomonas reinhardtii*. *Plant J* **32,** 467-480.

LIEN, T. & KNUTSEN, G. (1979). Synchronous growth of *Chlamydomonas reinhardtii (Chlorophyceae)* - Review of optimal conditions. *J Phycol* **15,** 191-200.

LILLY, J. W., MAUL, J. E. & STERN, D. B. (2002). The *Chlamydomonas reinhardtii* organellar genomes respond transcriptionally and post-transcriptionally to abiotic stimuli. *Plant Cell* **14,** 2681-2706.

LINCHAO, S., WONG, T. T., MCDOWALL, K. J. & COHEN, S. N. (1994). Effects of nucleotide-sequence on the specificity of RNA-dependent and Rnase E-mediated cleavages of RNA I-encoded by the pBR322 Plasmid. *J Biol Chem* **269,** 10797-10803.

LISITSKY, I., KLAFF, P. & SCHUSTER, G. (1996). Addition of destabilizing poly(A)-rich sequences to endonuclease cleavage sites during the degradation of chloroplast mRNA. *P Natl Acad Sci USA* **93,** 13398-13403.

MAGEE, A. M. & KAVANAGH, T. A. (2002). Plastid genes transcribed by the nucleus-encoded plastid RNA polymerase show increased transcript accumulation in transgenic plants expressing a chloroplast-localized phage T7 RNA polymerase. *J Exp Bot* **53,** 2341-2349.

MARTIN, W., RUJAN, T., RICHLY, E., HANSEN, A., CORNELSEN, S., LINS, T., LEISTER, D., STOEBE, B., HASEGAWA, M. & PENNY, D. (2002). Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *P Natl Acad Sci USA* **99,** 12246-12251.

MAUL, J. E., LILLY, J. W., CUI, L., DEPAMPHILIS, C. W., MILLER, W., HARRIS, E. H. & STERN, D. B. (2002). The *Chlamydomonas reinhardtii* plastid chromosome: Islands of genes in a sea of repeats. *Plant Cell* **14,** 2659-2679.

MAYFIELD, S. P., YOHN, C. B., COHEN, A. & DANON, A. (1995). Regulation of chloroplast gene expression. *Ann Rev Plant Phys* **46,** 147-166.

MERCHANT, S. S., PROCHNIK, S. E., VALLON, O., HARRIS, E. H., KARPOWICZ, S. J., WITMAN, G. B., TERRY, A., SALAMOV, A., FRITZ-LAYLIN, L. K., MARECHAL-

DROUARD, L., MARSHALL, W. F., QU, L. H., NELSON, D. R., SANDERFOOT, A. A., SPALDING, M. H., KAPITONOV, V. V., REN, Q. H., FERRIS, P., LINDQUIST, E., SHAPIRO, H., LUCAS, S. M., GRIMWOOD, J., SCHMUTZ, J., GRIGORIEV, I. V., ROKHSAR, D. S., GROSSMAN, A. R., CHLAMYDOMONAS, A. & TEAM, J. G. I. A. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **318,** 245-251.

MIYAMOTO, T., OBOKATA, J. & SUGIURA, M. (2004). A site-specific factor interacts directly with its cognate RNA editing site in chloroplast transcripts. *P Natl Acad Sci USA* **101,** 48-52.

MONDE, R. A., SCHUSTER, G. & STERN, D. B. (2000). Processing and degradation of chloroplast mRNA. *Biochimie* **82,** 573-582.

NICKELSEN, J. (2003). Chloroplast RNA-binding proteins. *Curr Genet* **43,,** 392 - 399.

NICKELSEN, J., DILLENWIJN, J., RAHIRE, M. & ROCHAIX, J. D. (1994). Determinants for stability of chloroplast *psbD* RNA are located within its short leader region in *Chlaydomonas reinhardtii*. *EMBO J* **13,** 3182-3191.

NICKELSEN, J., FLEISCHMANN, M., BOUDREAU, E., RAHIRE, M. & ROCHAIX, J.-D. (1999). Identification of *cis*-Acting RNA leader elements required for chloroplast *psbD* gene expression in *Chlamydomonas*. *Plant Cell* **11,** 957-970.

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

NICKELSEN, J. & LINK, G. (1993). The 54-kDa RNA-binding protein from mustard chloroplasts mediates endonucleolytic transcript-3' end formation invitro. *Plant J* **3,** 537-544.

PLANT, A. L. & GRAY, J. C. (1988). Introns in chloroplast protein-coding genes of land plants. *Photosynth Res* **16,** 23-39.

RACE, H. L., HERRMANN, R. G. & MARTIN, W. (1999). Why have organelles retained genomes? *Trends Genet* **15,** 364-370.

RIESMEIER, J. W., WILLMITZER, L. & FROMMER, W. B. (1994). Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *EMBO J* **13,** 1-7.

ROCHAIX, J.-D. (1996). Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. *Plant Mol Biol* **32,** 327-341.

ROTT, R., LIVEANU, V., DRAGER, R. G., STERN, D. B. & 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,** 307-314.

SAGER, R. & RAMANIS, Z. (1973). Mechanism of maternal inheritance in *Chlamydomonas* - biochemical and genetic studies. *Theor Appl Genet* **43,** 101-108.

SALVADOR, M., SUAY, L., ANTHONISEN, I. & KLEIN, U. (2004a). Changes in the 5'-untranslated region of the *rbcL* gene accelerate transcript degradation more than 50-fold in the chloroplast of *Chlamydomonas reinhardtii*. *Current Genetics* **45,** 176-182.

SALVADOR, M. L. & KLEIN, U. (1999). The redox state regulates RNA degradation in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Physiol.* **121,** 1367-1374.

SALVADOR, M. L., KLEIN, U. & BOGORAD, L. (1993a). 5' sequences are important positive and negative determinants of the longevity of *Chlamydomonas* chloroplast gene transcripts. *P Natl Acad Sci USA* **90,** 1556-1560.

SALVADOR, M. L., KLEIN, U. & BOGORAD, L. (1993b). Light-regulated and endogenous fluctuations of chloroplast transcript levels in *Chlamydomonas*. Regulation by transcription and RNA degradation. *Plant J* **3,** 213-219.

SALVADOR, M. L., KLEIN, U. & BOGORAD, L. (1998). Endogenous fluctuations of DNA topology in the chloroplast of *Chlamydomonas reinhardtii*. *Mol Cell Biol* **18,** 7235-7242.

SALVADOR, M. L., SUAY, L., ANTHONISEN, I. L. & KLEIN, U. (2004b). Changes in the 5 '-untranslated region of the *rbcL* gene accelerate transcript degradation more than 50-fold in the chloroplast of *Chlamydomonas reinhardtii*. *Curr Genet* **45,** 176-182.

SAMBROOK, J. & RUSSELL, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3er edn. edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SHIINA, T., ALLISON, L. & MALIGA, P. (1998). *RbcL* transcript levels in tobacco plastids are independent of light: Reduced dark transcription rate is compensated by increased mRNA stability. *Plant Cell* **10,** 1713-1722.

SHIINA, T., TSUNOYAMA, Y., NAKAHIRA, Y. & KHAN, M. S. (2005). Plastid RNA polymerases, promoters, and transcription regulators in higher plants. *Int Rev Cytol* **244,** 1-68.

SIEBURTH, L. E., BERRY-LOWE, S. & SCHMIDT, G. W. (1991). Chloroplast RNA stability in *Chlamydomonas*: rapid degradation of *psbB* and *psbC* transcripts in two nuclear mutants. *Plant Cell* **3,** 175-189.

SIMPSON, C. L. & STERN, D. B. (2002). The treasure trove of algal chloroplast genomes. Surprises in architecture and gene content, and their functional implications. *Plant Physiol* **129,** 957-966.

SINGH, M., BOUTANAEV, A., ZUCCHI, P. & BOGORAD, L. (2001). Gene elements that affect the longevity of *rbcL* sequence-containing transcripts in *Chlamydomonas reinhardtii* chloroplasts. *P Natl Acad Sci USA* **98,** 2289-2294.

SOLL, J. & SCHLEIFF, E. (2004). Protein import into chloroplasts. *Nat Rev Mol Cell Bio* **5,** 198-208.

STERN, D. B., JONES, H. & GRUISSEM, W. (1989). Function of Plasmid mRNA 3´ Inverted Repeats. *J Biol Chem* **264,** 18742-18750.

STERN, D. B. & KINDLE, K. L. (1993). 3' end maturation of the *Chlamydomonas Reinhardtii* chloroplast-*AtpB* Messenger-RNA is a 2-step process. *Mol Cell Biol* **13,** 2277-2285.

STERN, D. S., HIGGS, D. C. & YANG, J. (1997). Transcription and translation in chloroplasts. *Trends Plant Sci* **2,** 308-315.

SUAY, L., SALVADOR, M. L., ABESHA, E. & KLEIN, U. (2005). Specific roles of 5 ' RNA secondary structures in stabilizing transcripts in chloroplasts. *Nucl Acids Res* **33,** 4754-4761.

SUEOKA, N. (1960). Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardii*. *P Natl Acad Sci USA* **46,** 83-911.

SUGIURA, M., HIROSE, T. & SUGITA, M. (1998). Evolution and mechanism of translation in chloroplasts. *Annu Rev Genet* **32,** 437-459.

THOMPSON, R. J. & MOSIG, G. (1984). Light and genetic determinants in the control of specific chloroplast transcripts in *Chlamydomonas reinhardtii*. *Plant Physiol* **76,** 1-6.

TILLICH, M., LEHWARK, P., MORTON, B. R. & MAIER, U. G. (2006). The evolution of chloroplast RNA editing. *Mol Biol Evol* **23,** 1912-1921.

TROXLER, R. F., ZHANG, F., HU, J. & BOGORAD, L. (1994). Evidence that sigma-factors are components of chloroplast RNA-polymerase. *Plant Physiol* **104,** 753-759.

VAISTIJ, F. E., BOUDREAU, E., LEMAIRE, S. D., GOLDSCHMIDT-CLERMONT, M. & 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*. *P Natl Acad Sci USA* **97,** 14813-14818.

WEIHE, A. & BORNER, T. (1999). Transcription and the architecture of promoters in chloroplasts. *Trends Plant Sci* **4,** 169-170.

YOSHINAGA, K., IINUMA, H., MASUZAWA, T. & 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. *Nucl Acids Res* **24,** 1008-1014.

ZUKER, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucl Acids Res* **31,** 3406-3415.