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5'-UTR Structural requirements for stability of *rbcL* transcripts in the chloroplast of *Chlamydomonas reinhardtii*

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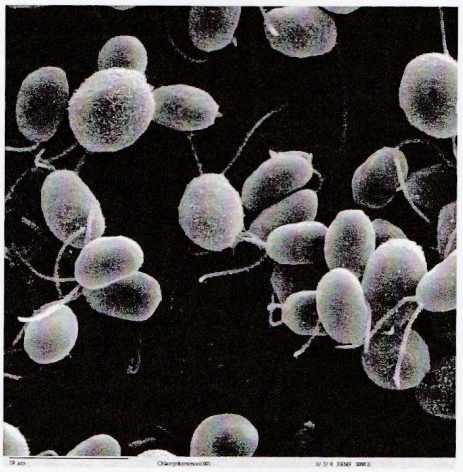

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Oslo, February 2003.

Alex Pastor Bernier.

Table of Contents

Abstract

The *rbcL* gene is constitutively expressed in the *Chlamydomonas* chloroplast.
The gene encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible for CO_2 fixation in photosynthesis.
The *rbcL* gene contains a 5' untranslated region (5' UTR) that harbours essential elements for regulation of gene expression.
A basic promoter element located at position –10 relative to transcription start and several downstream regulatory elements within the leader region have been recently characterized. Adding five extra nucleotides to the 5' terminus of *rbcL* transcripts decreased transcript stability significantly. The results suggest that sequence and/or conformational features are important for RNA stability in chloroplasts.

I. Introduction

1. *Chlamydomonas* as a model organism

Chlamydomonas reinhardtii is an unicellular green alga belonging to the group of chlorophycea. The genus *Chlamydomonas* (from greek chlamys monas, the solitary cloak) has been early established at the beginning of the nineteenth-century based solely on morphological criteria.
The genus *Chlamydomonas* consists of 459 recognized species (Harris, 1989).
Chlamydomonas are unicellular chlorophyte organisms harbouring two anterior flagella, a well-defined basal body, a distinct multilayered cell-wall and a basal chloroplast surrounding one or more pyrenoids. The pyrenoid is a distinctive body within the chloroplast engaged in CO_2 fixation and related dark reactions of photosynthesis. The number and distribution of pyrenoids has been a classic criterion in taxonomic distinction of the genus *Chlamydomonas* from other genera like *Chloromonas,* although taxonomical work based on molecular studies has shown evidences of great divergence among representatives in the genus and a close relationship with members of the genus *Volvox.*

Chlamydomonas reinhardtii is a soil and fresh water living organism with a wide distribution. First isolates date from soil extracts in Massachussets, 1945 (Harris, 1989).
The ukranian botanist Reinhardt, L. documented the first description of the species in 1888.
The organism shares many of the experimental benefits with the budding yeast *Sacharomyces cerevisiae* and emerges as a powerful tool for studying a large set of biological processes including photosynthesis, organelle biosynthesis, flagellar assembly and motility, phototaxis, circadian rhythms, gametogenesis and cellular metabolism.
Chlamydomonas is an eukaryote with a short generation time of about 5hrs and a well-known haploid genetics. Similar to yeast, *Chlamydomonas* has a sexual cycle. Meiospore formation is controlled by two mating-type loci, (mt +, mt -) and can be used for conventional tetrad analysis. Unlike yeast it harbours a single chloroplast and has a well-defined flagellar system.
Chlamydomonas offers a number of technical advantages too. It can be easily cultured in unexpensive media, on petri dishes or Erlenmeyer flasks and allows for genome transformation in both nucleus and organelles.

The organism is also very versatile with respect to nutrient requirements. *Chlamydomonas* can be grown in absence of light with an appropriate carbon source. Nonphotosynthetic mutants can be readily isolated and grown in the presence of acetate. This is a remarkable advantage that has been wisely employed in studying the photosynthetic electron transport chain. A wide scope of auxotroph mutants has been characterized as well (Harris, 2001).

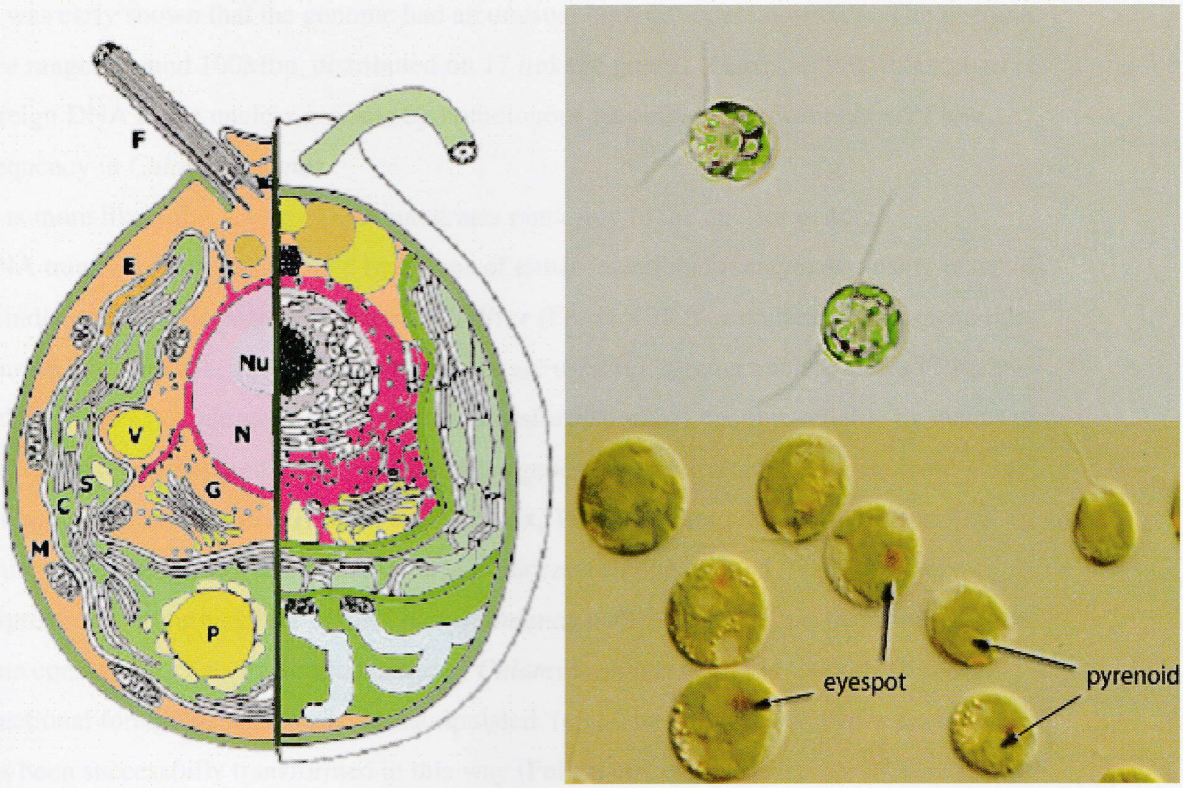


Fig 1.
***Chlamydomonas reinhardtii*, a model organism.**

Chlamydomonas is an unicellular alga of ~10µm diameter harbouring a pair of flagella that ranges from 10 to 12µm in length. The organism has a single cup shaped chloroplast that occupies two thirds of the basal cell and partially encloses the nucleus. It also has a carotenoid-containing body involved in phototaxis, an eyespot, a well-defined basal body and a distinct cellulose-lacking 7- layered cell wall.
The two and three-dimensional view of the cell shows the central nucleus (N), Nucleolus (Nu), the two isoform flagellae (F), the mitochondrion (M) and the cup-shaped chloroplast (C) including the eyespot (E) and the starch containing pyrenoid (P). Golgi vesicles (G), starch grains (S) and vacuoles (V) are also visible.

Nickelsen and Kück, 2000 (modified).

On the right, two views of *Chlamydomonas* under the light microscope.400x

www.rbgsyd.gov.au/information_about_plants/botanical_info/australian_freshwater_algae2/algpic/motile_microalgae
Botanic Gardens Trust. Sydney

2. The Nuclear Genome

As other photoautotrophic organisms, *Chlamydomonas* contains three autonomous genetic systems, which are localized in the nucleus, the chloroplast, and the mitochondrion.
The nuclear genome was the first to be characterized in *Chlamydomonas* (Sueoka, 1960).
It was early shown that the genome had an unusual high GC content of 62%. The genome size ranges around 100Mbp, distributed on 17 linkage groups (Harris, 1993). Integration of foreign DNA in the nuclear genome by homologous recombination occurs at very low frequency in *Chlamydomonas*.
It is more likely that foreign DNA integrates randomly in the nuclear genome.
DNA transformants are obtained by means of either insertional mutagenesis using plasmids (Kindle, 1998), transposable elements, *Gulliver* (Ferris, 1989) or mutant complementation (Purton and Rochaix, 1994). Those strategies are used for tagging nuclear genes (Tam and Lefebvre, 1993). *Chlamydomonas* nuclear transformation has become reliable by means of a "glass-bead" method and homologous marker genes such as argininosuccinate lyase (*arg7*) and nitrate reductase (*nit1*) (Purton and Rochaix, 1994). Techniques employing carbide whiskers and electroporation have been also successfully performed (Harris, 2001).
Expression of foreign genes in the *Chlamydomonas* nuclear genome has been shown to be time consuming. Since the codon usage of *Chlamydomonas* is biased towards G and C, functional foreign genes have to be manipulated. (e.g) The *green fluorescence protein,* GFP has been successfully transformed in this way (Fuhrmann, et al., 1999).
Intron processing and gene silencing are additional mechanisms to be taken into account regarding foreign gene expression (DEAH-box RNA helicase; Wu-Scharff, et al., 2000)
Molecular characterisation of mating-type loci have been early determined by chromosome walking (Ferris and Goodenough, 1994) and are not only important for understanding the mating behaviour of the cells but also for organelle inheritance processes.

Although controlled by two mating-type loci, the genetics of organelles and of the nucleus has been shown to be completely different. While nuclear mutations are inherited in a Mendelian fashion, an uniparental inheritance of chloroplast and mitochondrion genes is observed, depending on the mating type of the parent cells, that is mt + or mt –.

The *Chlamydomonas* nuclear genome displays a mendelian 2:2 ratio upon tetrad segregation, whereas chloroplast and mitochondrion segregate 4:0 (mt +) and 0:4 (mt -) respectively (Dron et al., 1983; Boynton et al., 1987), mendelian/non-mendelian inheritance (Sager, 1954).

A figure depicting *Chlamydomonas* organelle and nuclear inheritance is shown below.

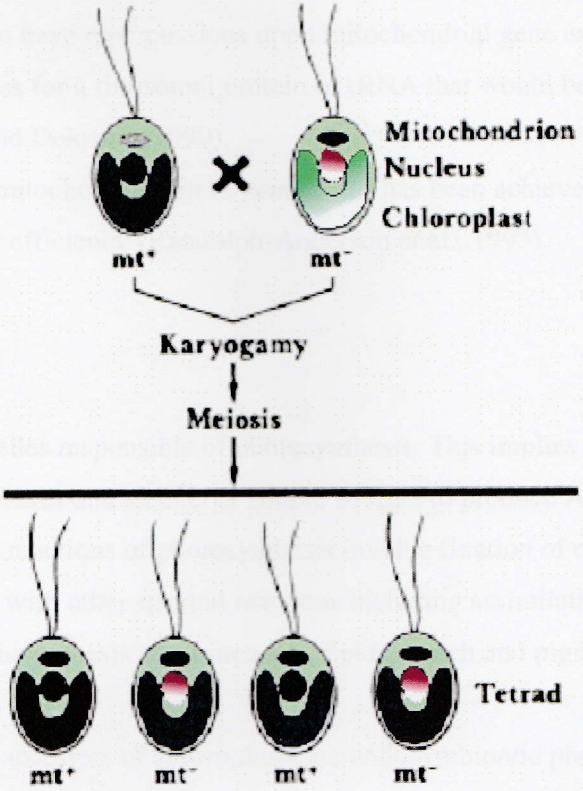


Fig 2.
Organelle inheritance in *Chlamydomonas*.

Chloroplasts appear either in green or grey. Nucleus is shown in pink or black. Mitochondrion is coloured in grey or black depending on the mating type. Uniparental inheritance is indicated in the first generation, first tetrad after meiosis. The maternal type is indicated only for the nucleus for simplicity, although the colour code allows us to follow the type of inheritance in each organelle.

Nickelsen and Kück, 2000 (modified).

3. The Mitochondrial Genome

The mitochondrion of *Chlamydomonas* is an organelle with a relatively small genome size compared to mitochondrial genomes of other green algae and land plants (Grant and Chiang, 1980). It consists of a 15.8kb linear DNA molecule and contains 7 respiratory genes, a reverse-transcriptase-like gene and only 3 tRNA genes (Gray and Boer, 1988; Michaelis et al., 1990). The other tRNAs have to be presumably imported into the mitochondrion, and there is some evidence that an organelle cross talk does exist.
(e.g) A chloroplast suppressor gene that acts on a missense mutation within the chloroplast *rbcL* gene has proven to have repercussions upon mitochondrial gene expression. It is likely that the suppressor codes for a ribosomal protein or tRNA that would be shared between organelles (Bennoun and Delosme, 1999).
Transformation of the mitochondrion in *C. reinhardtii* has been achieved successfully although with very low efficiency (Randolph-Anderson et al., 1993).

4. The Chloroplast

Chloroplasts are organelles responsible of photosynthesis. This implies the participation of thylakoid protein complexes and an energy source as light to produce ATP, a redox potential, and NADPH. The dark reactions of photosynthesis involve fixation of carbon to synthesize sugars and are coupled with other stromal reactions including assimilation of nitrogen, sulphur, phosphorus, biosynthesis of aminoacids, lipids, starch and pigments (Bogorad, 2000; Zerges, 2000).
The closest-free living ancestors of chloroplasts are endosymbiontic photosynthetic eubacteria probably of the lineage of cyanobacteria (Archibald and Keeling, 2002).
Most of the functions belonging to the ancestral chloroplast have been partially or totally relegated to the nucleus. Chloroplast gene expression is tightly regulated.
The circular chloroplast genomes differ among the species of algae and higher plants in size and copy number. Size ranges from 85kb in the siphonous green alga *Codium fragile* to 290kb in some representants of *Chlamydomonas* (Palmer, 1985).
Genome copy number per chloroplast has also a remarkable divergence, ranging from 80 in *C.reinhardtii* versus thousands in the mesophyll cells of higher plants (Zou, 2001).

The discovery and understanding of the *Chlamydomonas* chloroplast genome played a key role in the establishment of *Chlamydomonas* as a model organism (Rochaix, 2001).
From the 1970s to 1990s improved methods for mapping chloroplast genes were developed (Gillham, 1994) and by 2002 the first complete sequence was released (Maul et al., 2002).
The *Chlamydomonas* chloroplast genome consists of 203kb and contains 34 genes involved in photosynthesis, 31genes involved in both chloroplast transcription and translation, 1 protease gene, 29 tRNA genes and 9 genes of unknown function.
The genome is divided by two 21.2kb inverted repeats (IR_A, IR_B), harbouring gene clusters of rRNA and the photosystem II gene *psbA* (Maul et al., 2002).
This feature is reminiscent of higher-plant chloroplast organisation (Rochaix, 1978).
Chloroplast genes are mostly arranged in operon-like transcription clusters and such organisation is well conserved in land plants such as tobacco and maize, whereas extensive gene rearrangements are observed in algae (Rochaix, 1997).
Additionally, algae chloroplast genomes are usually larger than their land counterparts.
The presence of numerous intron sequences, enlarged spacers and high repetitive content in DNA is responsible for large algal genome sizes (Boudreaux et al., 1994).

Biolistic transformation systems were early developed in *C. reinhardtii* and multiple strategies to selectively clone foreign DNA in the chloroplast were subsequently performed (Boynton et al., 1988).
Homologous recombination has proven to be successful and a number of mutants has been obtained since then by means of gene-complementation (Purton and Rochaix, 1994) or using other selective markers such as antibiotics, *aadA* (Goldschmidt-Clermont, 1991), *AphA-6* (Bateman and Purton, 2000), *uidA* (GUS) (Salvador et al., 1992), *Renilla* luciferase (Minko et al., 1999).

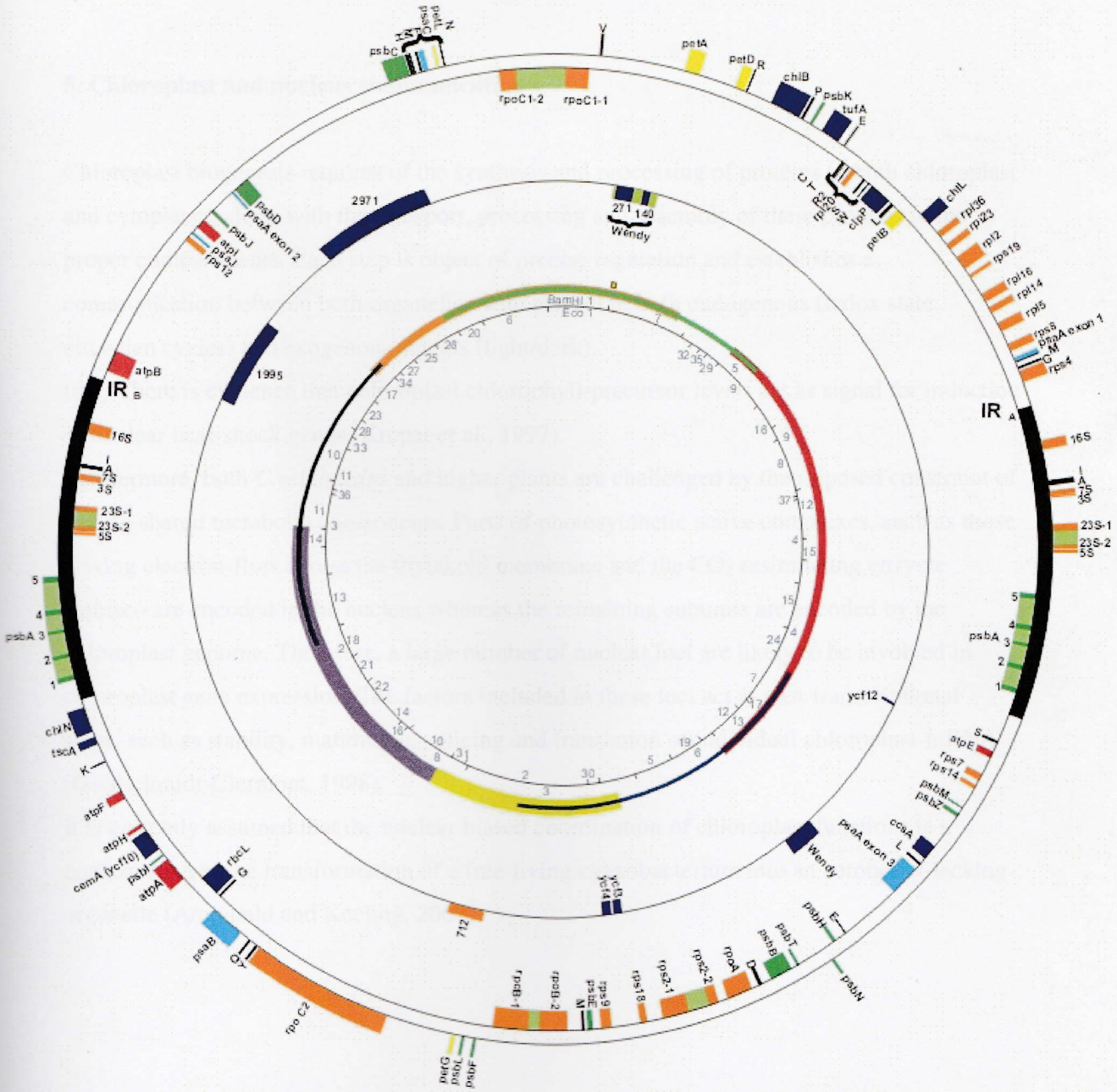


Fig 3.

The *Chlamydomonas* Chloroplast Genome.

The inner circle displays *BamHI* and *EcoRI* restriction fragments. The next interior circle shows seven overlapping BAC clones spanning the genome. The immediate exterior circle shows open reading frames, ORFs along with genes of unknown function. The outer circle shows genes with known or expected function. Introns are in olive green. Two large inverted repeats IR_A and IR_B harboring the *psbA* gene and chloroplast ribosome components are depicted in black.

http://bti.cornell.edu/bti2/chlamyweb/default.html

5. Chloroplast and nucleus communication

Chloroplast biogenesis requires of the synthesis and processing of proteins in both chloroplast and cytoplasm, along with the transport, processing and assembly of these proteins in the proper compartments. Each step is object of precise regulation and establishes a communication between both organelles with respect to both endogenous (redox state, circadian cycles) and exogenous signals (light/dark).

(e.g) There is evidence that chloroplast chlorophyll-precursor levels act as signal for induction of nuclear heat-shock genes (Kropat et al., 1997).

Furthermore, both *C.reinhardtii* and higher plants are challenged by the imposed constraint of having shared metabolic components. Parts of photosynthetic active complexes, such as those driving electron-flow across the thylakoid membrane and the CO_2 assimilating enzyme Rubisco are encoded in the nucleus whereas the remaining subunits are encoded by the chloroplast genome. Therefore, a large number of nuclear loci are likely to be involved in chloroplast gene expression. The factors included in these loci act at post-transcriptional steps, such as stability, maturation, splicing and translation of individual chloroplast-mRNA (Goldschmidt-Clermont, 1998).

It is currently assumed that the nuclear biased coordination of chloroplast functions is a consequence of the transformation of a free-living cyanobacterium into an autonomy-lacking organelle (Archibald and Keeling, 2002).

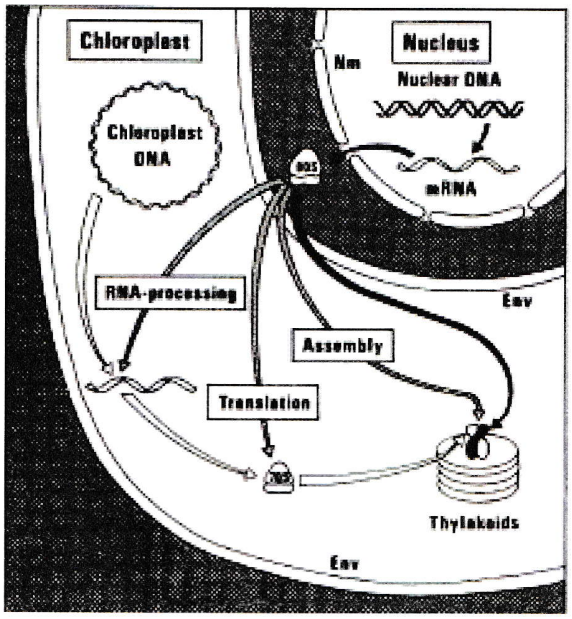


Fig 4.

Chloroplast/Nucleus communication.

Photosynthetic multisubunit complexes are formed by both nuclear encoded (black arrows) and chloroplast encoded (white arrows) polypeptides. Gray arrows indicate nuclear factor mediated regulation points.

Nickelsen and Kück, 2000.

6. Transcription and regulation of transcription in chloroplast genes

Studies on plastid mRNA accumulation in a number of species have shown that mRNA levels, which are generally determined by the transcription rate and mRNA stability, fluctuate in response to both developmental and environmental signals (Salvador et al., 2004). Transcription of individual chloroplast genes is mainly directed by at least two distinct RNA polymerases, PEP and NEP respectively.

• PEP (Plastid Encoded Polymerase) resembles bacterial RNA polymerases.
PEP recognizes prokaryotic-like chloroplast promoters typically containing the –35 TTGACA and –10 TATAAT consensus elements (Kim et al., 1999).

• NEP (Nuclear Encoded Polymerase) is a monomeric enzyme with some similarities with bacteriophage and mitochondrial encoded polymerases
(Hajdukiewicz et al., 1997; Cahoon et al., 2001).

NEP-promoters are found in a variety of chloroplast genes as those encoding for rRNAs (e.g. rrn16 gene in tobacco), ribosomal proteins and other 'house-keeping' genes involved in the basic metabolism of chloroplast.
However, NEP promoters are practically absent in those genes involved in photosynthesis (Hajdukiewicz et al., 1997; Kapoor et al., 1997).

Each RNA polymerase recognizes particular promotor-elements through the interaction of sigma-like factors or additional nuclear encoded factors (Allison and Maliga, 1995; Christopher et al., 1992).
Recent studies suggest that PEP requires only the –10 basic promotor element.
The –35 element is shown to be dispensable for promotor activity in a number of genes as shown in blue-light responsive *psbD* promoter in barley (Kim et al., 1999), spinach tRNA (Gruissem and Zurawski, 1985), and *Chlamydomonas rbcL* and *atpB* genes (Klein et al., 1992). In fact, *Chlamydomonas rbcL* gene seems to be transcribed through the intervention of a downstream enhancer-element and unidentified enhancer-binding proteins (Anthonisen et al., 2002).

There is evidence that the relative activities of the different RNA polymerases are influenced by both light and developmental signals (Allison, 2000). In general, chloroplast genes are transcribed by NEP during the early stage of plant development whereas the following transcription of photosynthesis-related genes are contributed by PEP
(Kapoor et al., 1997; Mullet, 1993).

The fluctuations of the transcription levels of plastid genes seem to be responsive to plastid development, plastid type, cell cycle and environmental factors such as circadian cycles. Transcription levels may correlate with endogenous fluctuations of DNA topology, which suggests the presence of chromatin-modification processes. *psaB, rbcL* and *atpA* genes are a group of *Chlamydomonas* chloroplast encoded genes displaying tightly associated transcription rates with circadian controlled chromatin packing processes
(Salvador et al., 1998).

Surprisingly, relative transcription rates of many individual genes are constant compared to the overall dramatic changes upon transcription. This suggests that the regulation of transcription is not a limiting step in chloroplast gene expression, while posttranscriptional events including RNA processing, RNA stability, translation rate and protein turnover may play an important role in differential chloroplast gene expression (Mayfield et al., 1995).

7. Post-transcriptional processes in the *Chlamydomonas* Chloroplast

7.1 RNA stabilisation and degradation, 5' and 3' RNA stability determinants

Transcript half-live ranges from minutes in prokaryotes to hours in chloroplasts.
Regulation of RNA transcript-accumulation rates accounts for changes between relative transcription and degradation rates. The steady-state level of a given RNA is determined by the rate of its synthesis and degradation.
Transcript-specific nuclear-encoded RNA binding proteins seem to protect RNA against nucleolytic attack in chloroplasts by interaction with cognate sequence elements (Nickelsen, 1998). These factors are denominated nuclear trans-acting factors or more generally trans-associating factors. The introduction of site-directed gene mutations in the chloroplast revealed that both 5' and 3' untranslated regions (UTRs) contain elements required for transcript stability.
There is strong evidence that the 5'UTR in *psbB, psbD* and *petD* is required for transcript stabilisation in *Chlamydomonas* and that the region includes cognate elements for nuclear trans-acting factors. However, not only the transcript sequences within the 5' and 3'UTR are involved in transcript stabilisation. The RNA secondary structures are also essential (Nickelsen et al., 2000; Vaistij et al., 2000; Nickelsen, 2003).

7.2 5'UTR transcript stabilisation

The *Chlamydomonas* chloroplast *psbB* 5'UTR has been mapped as the stability determinant of *psbB* mRNA and the target site for Mbb1, a nuclear encoded trans-acting factor (Monod, et al., 1992). Mutant analysis in *Chlamydomonas* has thrown some light on the function of trans-acting factors.

Mbb1 function is likely to be required in the processing or stabilisation of mature *psbB* mRNAs with a shorter 5'UTR. The nuclear Mbb1 gene has been cloned, and the encoded protein isolated and identified.
Mbb1 is a chloroplast stromal protein that is part of a 300kDa RNA-associating protein complex, which is most likely to be involved in *psbB* mRNA processing, stability and translation. It has been suggested that mRNA processing could be mediated by 5'-3' exoribonuclease activity undertaken by this complex (Vaistij et al., 2000).

A similar mechanism seems to apply for the chloroplast *psbD* mRNA as shown by *nac2* and *petD* nuclear mutant analyses (Kuchka et al., 1989; Zerges, 2000).
For the *psbD* gene, two major upstream cis-elements conferring RNA stability have been identified along with the binding site for a potentially stabilising factor, encoded by the *nac2* gene (Nickelsen, 1999). The product of *nac2* gene is also a RNA-associating protein complex that localizes in the chloroplast stroma (Boudreau, et al., 2000). A basic mechanism for *nac2*-mediated transcript stabilisation has been suggested (Nickelsen, 2003). ***Section IV, 2***.

In some chloroplast genes the specific cis-elements conferring RNA stability are embedded downstream of the transcription start.
This is the case of a 10nt element in *atpB* gene (+31 to +42) and a 12nt element (+38 to +47) in the *rbcL* gene. In both genes the 5'UTR stabilising elements are predicted to be part of RNA secondary structures, although the structural features alone do not seem to be responsible for the longevity of the transcripts (Anthonisen et al., 2001).

There is also evidence for specific elements in the 5'UTRs being related to environmentally controlled gene expression.
The sequence spanning positions +21 to +41 in *Chlamydomonas rbcL* 5'UTRs sequence is required for photo-accelerated degradation of *rbcL* transcripts, which may be the target to light-induced endonucleases. Transcript analysis of GUS/*rbcL* chimeric genes revealed as much as 16-fold increase in degradation rates (Salvador et al., 1993b; Singh et al., 2001). Additional determinants for RNA stability in light-induced transcript degradation have been identified in the *rbcL*-coding region (+329 to +334) (Salvador et al., 1993b; Singh et al., 2001).

In higher plants, the determinants for chloroplast RNA stability have also been shown to reside in the 5' untranslated regions, specially by in vivo analysis via tobacco chloroplast transformation (Shiina et al., 1998; Eibl et al., 1999). However, it has been also reported that stability determinants can be embedded in the coding region, as it is the case of spinach chloroplast genes (e.g) *psbA*, (Klaff, 1995; Klaff and Gruissem, 1991).

8. Regulation of mRNA transcript stability

8.1.1 Developmental and environmental signals

Chloroplast mRNAs accumulate to different steady-state levels during development or in response to environmental signals.
Generally, the fluctuations in transcription rates alone do not account for changes in mRNA accumulation. This implies the contribution of mRNA stabilisation processes (Deng et al., 1987). For instance, changes in plastid mRNA stability in higher plants account for the differences in mRNA accumulation during leaf development (Klaff and Gruissem, 1991).

8.1.2 Light/Dark cycles

In the *Chlamydomonas* chloroplast, RNA half-lives of examined genes vary remarkably. There is a general tendency for increased stability in the dark for cells grown in a light/dark regime. (e.g) *rbcL* mRNA stability increases 6-fold in the dark relative to stability in the light suggesting the participation of light-accelerated degradation processes.
Similar examples have been found for regulation of *rbcL* mRNA stability in tobacco and barley (Shiina et al., 1998; Kim et al., 1993).

8.1.3 Redox State

Redox processes have shown to be related to RNA-turnover in chloroplasts (Salvador and Klein, 1999). Reducing agents (e.g) dithiothreitol, (DTT) reduce transcript stability in light whereas oxidative agents have a stabilizing effect.
Furthermore, interruption of the electron transport chain of photosynthesis by herbicides (e.g) 3-(3-4-dichlorophenyl)-1,1-dimethylurea, (DCMU) did also stabilize transcripts in light. This suggests that chloroplast proteins as redox-carriers may be involved in light/dark mediated RNA turnover (Salvador and Klein, 1999).

8.2 Trans-associating factors

8.2.1 Non proteinaceous trans-acting factors

In higher plants and in *Chlamydomonas*, chloroplast RNA stability can be affected by magnesium ions (Klaff, 1995; Nickelsen et al., 1994).
In-vitro chloroplast degradation of spinach *psbA* and *rbcL* mRNAs, 16S rRNA and (His)-tRNA is influenced by the concentration of Mg^{2+} ions.
The concentration of free Mg^{2+} rises during chloroplast development within a range sufficient to mediate stabilisation. It has been also observed that magnesium ions have the ability to sequester endonucleolytic cleavage sites of mRNA and also influence protein binding to mRNA-UTRs (Horlitz and Klaff, 2000).

8.2.2 Chloroplast RNA-binding ribonucleoproteins, cpRNPs

Besides the aforementioned trans-associating factors, there is evidence of protein factors that bind directly or cooperatively to specific sequence elements and may form part of large protein complexes involved in a multiple-stage RNA post-transcriptional regulation. The majority of these factors have been characterized in ribosome-free mRNAs and localize essentially in the stroma of chloroplasts (Nakamura et al., 2001).

8.2.3 Ribosome association

There is an interesting relationship between ribosome association to mRNA and transcript stabilisation.
In higher plants, organelle-specific translation inhibitors affecting polysome distribution have been used in *rbcL* and *psbA* transcript-decay assays. The observations suggested that gene transcripts for both genes were less stable in polysome-bound than polysome-depleted DNA (Klaff and Gruissem, 1991). In contrast, *rbcL* maize-transcript stability seems to correlate positively with polysome association (Barkan et al., 1993).
Similarly, *Chlamydomonas psbA* transcript stability also seems to increase when RNA is bound by ribosomes. It has been suggested that mRNA ribosome association affects the secondary structure in the 5'UTR and consequently affects transcript stability (Yohn et al., 1996; Bruick and Mayfield, 1998).

There is also some evidence of other protein complexes binding to UTR regions coupling translation and RNA processing/stabilisation.
(e.g) A protein complex assembling on the *psbA* 5' UTR has proven to couple translation initiation and photosynthetic activity (Nickelsen, 2003). The complex is integrated by 4 subunits: A Poly-A binding protein (cPABP), a disulfide isomerase, and RB47 an RNA binding protein.

Studies in *Nicotiana tabaccum* for *rbcL* chimeric genes display an interesting connection between transcript stability and translation. Transgenic tobacco lines containing different combinations of 5' UTR show a five-fold variation in *uidA*-mRNA transcript stability and 100-fold variation in GUS activity, a measure of translation activity. Posttranscriptional and translational processes seem to work particularly and coordinately from gene to gene (Eibl et al., 1999).

In other higher plants, like the C_4 plant *Amaranthus hypochondriacus*, a role for 5'UTRs in posttranscriptional and translational processes has been reported.
Here, a p47 nuclear-encoded factor correlates with polysome assembling at the 5' end of processed mRNAs. The transcript association of p47 factor is a light induced process mediating both transcript stabilization and translation initiation (McCormac et al., 2001).

9. The Scope of this Study

- *rbcL* gene

rbcL is a constitutively expressed gene in *Chlamydomonas* chloroplast.
The gene encodes the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme responsible for CO_2 fixation in photosynthesis.
rbcL contains a 5' untranslated region (5' UTR), which harbours essential elements for regulation of gene expression and a basic promotor element. The expression of *rbcL* is constitutive. The average stability of *rbcL* transcripts is about 20 hours in the dark.

- *rbcL* 5'UTR elements

Several elements spanning the *rbcL* 5'UTR that are involved in transcript stability have been characterized.
A putative 10 bp sequence extending from positions +38 to +47 relative to the transcription start is crucial for transcript global turnover (Anthonisen et al., 2001). Further deletion analysis spanning this region from position +36 in 3' to 5' direction confirmed the results (Salvador et al., 2004). Deletions in the 5' UTR spanning positions from +14 to +27 are also relevant for transcript stability (Singh et al., 2001).
It has been also reported that the sequence elements in 5'UTR regions of other chloroplast-encoded genes play an important role in the global transcript stability both in *Chlamydomonas* and in tobacco (Higgs et al., 1999; Zou et al., 2003).

It might be a connection between the sequence elements in transcript 5' UTRs and nuclear protein factors such as the cpRNPs (*Chloroplast RNA binding ribonucleoproteins*).
Changes affecting RNA secondary structure correlate generally with transcript stability.
This suggests that some positions in the RNA fold could be recognized by concrete protein motifs, possibly forming part of catalytic sites (Nickelsen and Kück, 2000; Vaistij et al., 2000; Nickelsen, 2003). Computer-based thermodynamic simulations of RNA secondary structures have been performed (Zuker et al., 1999) and further RNA structural analysis undertaken by treating RNA with dimethyl sulfate, (DMS) (Anthonisen et al., 2002).

The most likely transcript secondary structures for the first 152bp of the *rbcL* 5'end are depicted in **Fig 5.** This has to be regarded with caution because structural-analysis are not performed in-vivo. Data on secondary RNA structures are applied to RNA folding in-vitro, and without the participation of potential factors assisting RNA folding in-vivo. It remains to be proven that the given structures fit with the expected binding sites of cpRNPs.

- The +5bp insertion construct

5 extra bases, (GCGCG) have been added to the 5'end of the 5'UTR in *Chlamydomonas* chloroplast *rbcL* gene.
The extension changes the secondary structure at the 5' end of the transcript.
Analysis of the transcript levels in chloroplast transformants harbouring the modified sequence should reveal wether the structure at the 5'end of *rbcL* 5'UTR is important for RNA stability.

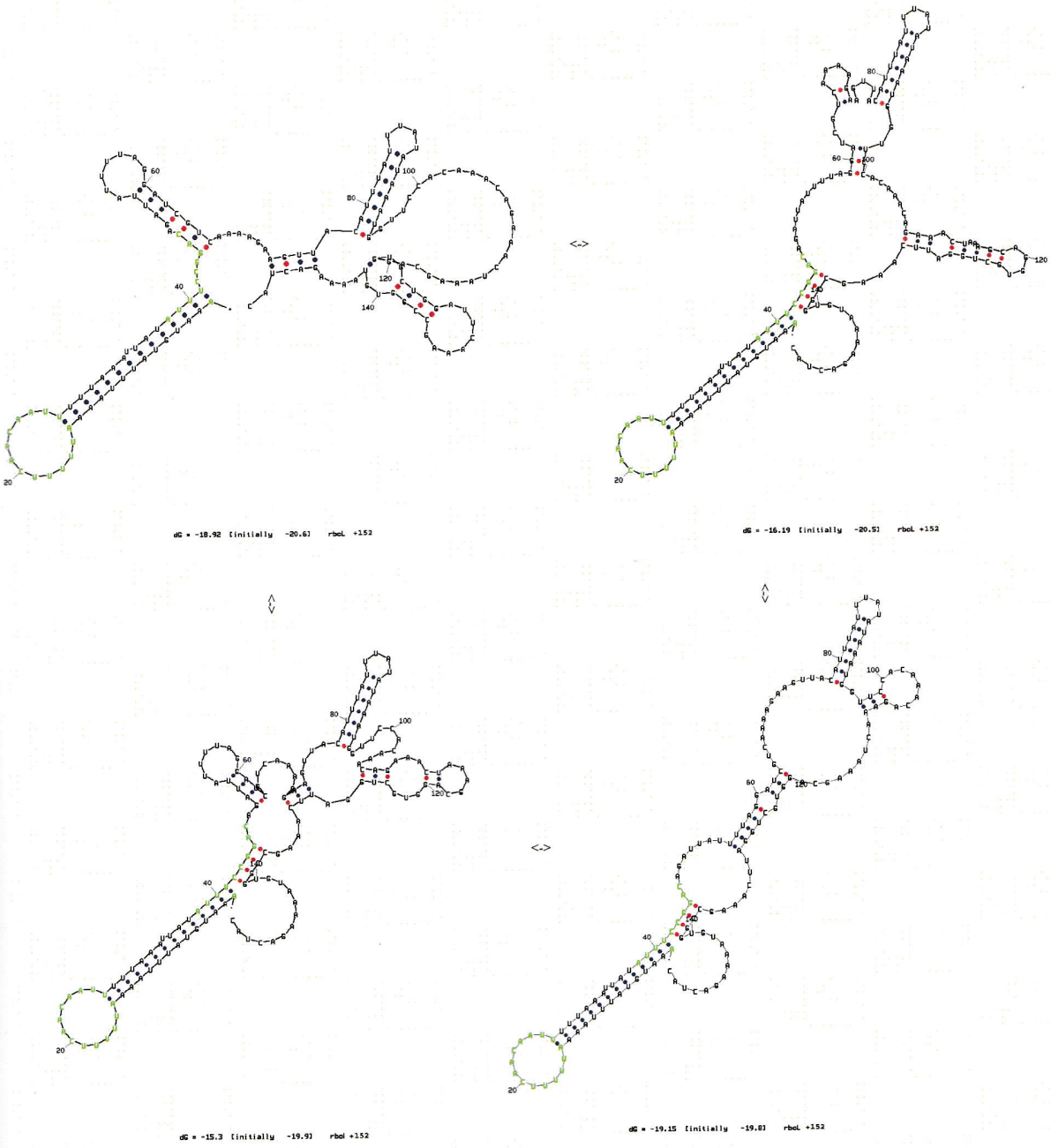


Fig 5.
Secondary structure thermodynamic simulations.
Folding models for bases +1 to 152 relative to transcription start in *rbcL* sequences.

The structures shown were selected according to thermodynamic optimisation criteria and display minimum Gibbs-formation energy. The red and blue squares indicate probable internal base pairing.
Note the green-labelled regions consisting of transcript stabilizing elements, positions +38 to 47 in the stem and +14 to 27 in the loop. Transcription start nucleotide, A, is preceded by a black dot and appears also in green.
The most-likely structure in-vitro may oscillate between several low-energetic structures depending on environmental conditions.

Software developed by Zuker et al., 1999 **(www.bioinfo.rpi.edu/applications/mfold/old/rna).**

II. Materials and Methods

1. Strains and Media

1.1 *Chlamydomonas reindhardtii*

All strains of *C. reinhardtii* used in this study were obtained from the *Chlamydomonas* Genetic Center at Duke University, NC.
The DNA constructs described below were transformed into the strain ac-uc-2-21 mt^+ (CC373), a non-photosynthetic mutant lacking a 2.5kb segment in its *atpB* coding region that extends into the inverted repeat (Boynton et al., 1991).
The mutant was grown in HSHA (High salt high acetate medium) in the dark (Harris, 1989) and transformed by means of microprojectile bombardment (Boynton et al., 1988).

1.1.1 Cultures

Transformants can be selected under light conditions on agar- HS plates, High salt media (Harris, 1989) and are first grown in 100mL Erlenmeyer flasks at room temperature and later transferred to either 150 or 250 ml tubes in a 32°C water bath.
The cultures were continuously bubbled with 2% CO_2 enriched air and diluted daily with fresh HS medium.
Cell cultures were grown under 12hr light/dark conditions for RNA isolations and transcription assays.

1.2 *Escherichia coli*

Escherichia coli strain TB1 is a prokaryote strain that has been used extensively for cloning. The cells were grown in LB medium as described in Sambrook et al. 2001.
Plasmids were stored in glycerol/LB frozen cell inoculates at –80°C. DNA material like oligos, maxi/mini-preparations, PCR products and enzymatically modified DNA were kept in 70% ethanol or dH_2O at -20 °C.
Two kinds of plasmids were used in this study, a moderate length plasmid SK+ was used to perform all DNA manipulations and a larger plasmid, MU21was used as the chloroplast transformation vector.

2. Plasmids

2.1 SK+157

SK+157 is a 5.2kb pBluescript SK+ based plasmid (Stratagene).

pBluescript SK+ has multiple cloning sites, ampicillin and β-galactosidase genes allowing for antibiotic selection. SK+ 157 also incorporates a chimeric 2.5kb construct whose elements are shown below.

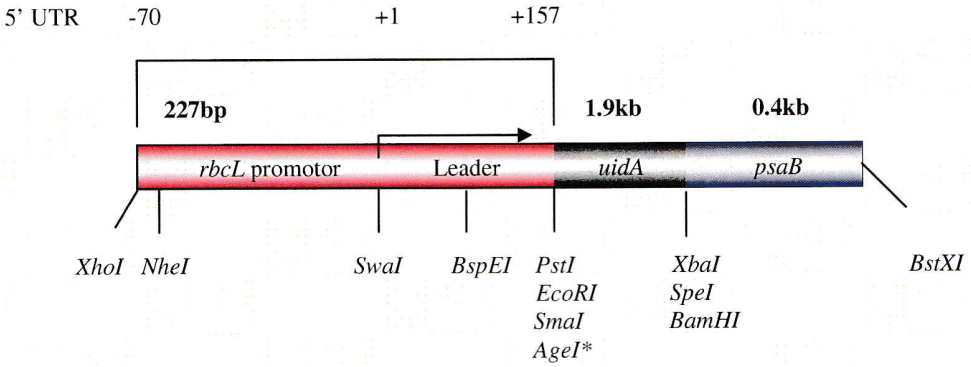


Fig. 6.

***rbcL/GUS/psaB* construct consisting of *rbcL* 5'UTR region and harbouring promotor and leader region (5'UTR downstream of transcription start, labeled as +1), *uidA* (GUS) gene and *psaB* terminator.**

Transcription occurs towards *uidA* and the *psaB* termination sequence as indicated by the bold arrow.
The size of the diverse components in the construct are indicated for each region in bold.
Restriction sites are shown below. All sites, except *SwaI* and *BspEI* were introduced in previous cloning steps (Not drawn to scale)

The construct contains the *rbcL* 5'UTR, the untranslated region of the *rbcL* gene, the *uidA*(GUS) coding region, a prokaryotic gene (*E. coli*) that encodes the enzyme β-glucuronidase and the 3'end of the *psaB* gene.

2.2 MU21 and modified vector, p +5

pMU21 is a 10.5 kb plasmid harbouring an *rbcL/uidA(GUS)* chimeric construct flanked by *atpB* chloroplast regions. This includes a complete functional *atpB* gene that is used as a selective marker in mutational restitution of *Chlamydomonas* transformation strain ac-uc-21 (CC-373) and IR, inverted repeat region. p+5 is the modified version of pMU21 harbouring 5 extra nucleotides at the 5' end of the UTR.

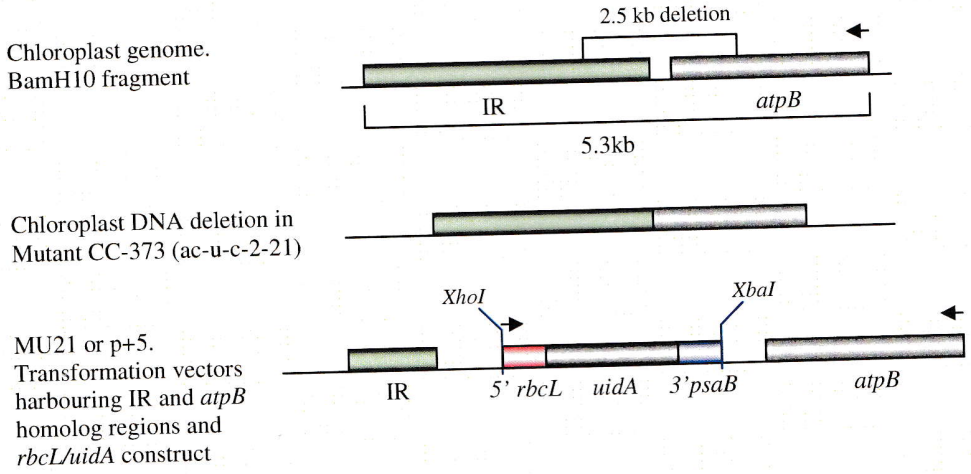


Fig. 7.

Schematic representation of the chloroplast transformation system used in this study

The target for recombination is CC-373 IR/*atpB* deleted region in mutant CC-373.
The p+5 plasmid is depicted below along with its components.
The direction of transcription is indicated by bold arrows.

3. Standard molecular techniques

- Restriction enzymes

Restriction enzymes were obtained and used for cloning as specified from suppliers New England Biolabs and Promega.

- Isolation of DNA fragments by gel electrophoresis

Enzyme digested DNA molecules were separated on 1% agarose gels.
In order to isolate the fragment of interest, the gel was examined under UV-light and a narrow well was made just below the band of interest. A piece of dialysis-membrane was damped shortly in TAE buffer, cut to the size of the well and smoothly dragged into the hole. The well was filled with TAE buffer. Gel electrophoresis was resumed and the DNA was collected in the well. DNA was precipitated and extracted from salts in TAE and ethidium bromide, as described in Sambrook and Russell, 2001.

- Isolation of plasmids from *E. coli*

Alkaline lysis followed by phenol extraction and ethanol precipitation methods were used to obtain plasmid DNA from *E. coli* cultures in small scale (minipreps).
To isolate plasmid DNA at large scale, the CsCl ethidium bromide gradient centrifugation method was employed (Sambrook and Russell, 2001)

- DNA and RNA quantifications

DNA and RNA quantitations were quantified by measuring the absorbance at 260nm (Spectrophotometer Shimadzu UV-1601) or by means of the dot spot method (Sambrook and Russell, 2001).

- Ligations

Blunt- and sticky-end ligations were performed with T4 DNA ligase according to the supplier's protocol (NEB).

- *E.coli* Transformations

Transformations were performed using *E.coli* $CaCl_2$ competent cells and the heat shock procedure as described in Sambrook and Russell, 2001.

- Oligo cloning

The synthetic oligos 5'*rbcL*+5 and 3'*rbcL*+5 were obtained from MWG Biotech (Germany) and were delivered in a dried state. They consist of a 46 and 50 bp complementary ssDNA spanning the *rbcL* region +1 to +45 relative to transcription start position.

· Sequences

5'*rbcL*+5 5′-GCGCGAAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTT-3′

3'*rbcL*+5 3′-CGCGCTTTACATAAATTTTAAAAAGTTGTTAAAAATTTAATATAAAGGCC-5′

· Oligo annealing and phosphorylation steps.

The oligos were resuspended in sterile distilled water to a concentration of 100µM.

Both oligos were joined in a quantity of 5nmol of each oligo were mixed and heated for 2 min at 100°C. The solution was cooled down to room temperature and phosphorylation of 5' ends was carried out by T4 polynucleotide kinase in the presence of ATP.

The double-stranded oligos were purified on a 1.3% agarose gel, isolated from the gel and purified by phenol/chloroform extraction and precipitated in 70% ethanol.

Concentrations were determined by the dot spot method according to supplied protocol.

· Oligonucleotide ligation on vector plasmid.

The oligonucleotide was ligated into the *SwaI/BspEI* sites of the vector p+157

- Sequencing

Plasmids were sequenced by the chain termination method using a GUS 3'-5' primer (Sanger et al., 1977).

- Chloroplast transformation in *Chlamydomonas reinhardtii*

Chlamydomonas atpB- mutant strain cells ac-uc-2-21 mt+, (CC-373) were transformed using micro projectile bombardment with DNA-coated 0.6 μm gold particles using a PDS-1000/He particle delivery apparatus, *BIORAD-Hercules, CA* (Boynton et al., 1987; Blowers et al., 1989). Upon transformation, the foreign DNA integrates into the chloroplast chromosome by homologous recombination and complements the sequences missing in the mutant, hence restoring photosynthetic capacity of the cells.
After transformation, the cells were kept in the dark overnight and transferred to HS-agar plates the next day. Transformants were selected for the restoration of *atpB* function, i.e. photosynthesis, and then analysed for the presence of the *uidA* gene (GUS).

- Isolation of genomic DNA

The transformants were grown in liquid HS media in the presence of light under continuous bubbling with CO_2-enriched air in a temperature controlled water bath at 32°C. DNA was isolated from the cultures (Dellaporta et al., 1983).

- Isolation of RNA

Cultures for RNA isolation were from under the same conditions as described for DNA isolation but illumination was set up to 12h light/dark cycles.
RNA was isolated twice, at 11h in the dark and after 1h in the light.
Isolation was performed according to the SDS/phenol method (Merchant and Bogorad, 1986).

- DNA slot blot

Samples containing 500ng of isolated DNA were denatured with NaOH and transferred to Zetaprobe nylon membrane (Biorad), using a slot blot apparatus (Hoefer Scientific Instruments, PR600) as described in Sambrook and Russell, 2001.
The membrane was hybridized to a labeled GUS probe, washed as described in the Biorad protocol and exposed to a Kodak Biomax MS film at –80°C for 12h.

- Southern Blot

Samples of *Chlamydomonas* chloroplast DNA containing 1.5µg were preheated in 65°C water bath and digested with enzymes *HindIII* and *KpnI* (New England Biolabs, NEB).
The DNA was separated on a 1% agarose gel and transferred to a Zetaprobe nylon membrane (Biorad) by alkaline capillary blotting as described in the Zetaprobe manual.
Membranes were hybridized with a labeled *atpB* probe and washed as described in the Biorad protocol and exposed to a Kodak Biomax MS film at –80°C overnight.

- Northern Blot

Samples of *Chlamydomonas* chloroplast total RNA containing ca. 4µg were dissolved in a formamide/formaldehyde solution, heated for 15min. at 65°C and then separated on a 1,3% agarose/formaldehyde gel (Sambrook and Russell, 2001). All materials used in this procedure were DEPC treated or RNase free. Formamide and formaldehyde chemicals are used to disrupt internal base pairing and secondary structures before and upon gel electrophoresis. RNA was transferred to a nylon membrane by alkaline capillary blotting as described in the Biorad Zetaprobe protocol. The membrane was hybridized to the *uidA* (GUS) labeled probe, then washed and exposed as in Southern blots.

- Probes

The *uidA* (GUS) probe was obtained by digesting vector pBI221 with the enzymes *SacI* and *BamHI* (Jefferson, 1987). DNA fragments were isolated by gel electrophoresis as described previously and labeled with (α-^{32}P)-[dCTP] by random primer labeling using the Klenow fragment of *E.coli* DNA polymerase I (Sambrook and Russell, 2001).

The *atpB* probe was obtained by digesting vector pCrcatpB with *EcoRV* and *HpaI* restriction enzymes (Blowers et al., 1990). The DNA fragments were isolated and labeled as the GUS probe.

- In-vivo determination of transcription rates

The [^{32}P]-Orthophosphate labeling technique is described by Baker et al, 1984 and Blowers et al, 1990. The relative rates of transcription of chimeric *rbcL*/GUS genes, the endogenous *rbcL* and *atpB* genes were determined by measuring the incorporation of [^{32}P] into GUS transcripts (Baker et al., 1984; Blowers et al., 1990).
The autoradiogram signals were quantified using 1D image analysis software (Kodak Digital Science, Rochester N.Y). Ratios of transcription rates were calculated from the increase in signal intensity at time points 10 min and 20 min and subsequently corrected for the lengths of the transcripts and normalized to the intensity of transcripts of the endogenous *rbcL* gene.

III. Results

1. Plasmid Cloning

Preliminary cloning steps

- Amplification of *rbcL* 5'UTR element in plasmid +157

A short region spanning –70 to +157 positions of *rbcL* 5'UTR was amplified by means of PCR using the primers.

5′ TCTATG*CTCGAG*TGATAAGACAAGTACATAAATTTGCTAGCTTACA 3′

5′ GTAGTAT*GTTAAC*CGGTAGTC 3′

The positions denoted in italics are inserted restriction sites (*XhoI*, in red. *HpaI*, in blue) that were subsequently used in cloning of the 5'UTR amplified sequence into the *XhoI/SwaI* sites into plasmid pCrc32 resulting in plasmid +157 (Anthonisen et al., 2001).

- Cloning of *rbcL* 5'UTR element in pBluescript SK+

The *rbcL/uidA* cassette harbouring *rbcL* sequences from positions –70 to +157 in plasmid +157 was subsequently cloned into the *XhoI/XbaI* sites of pBluescript SK+ giving SK+ 157, the starting plasmid for cloning in this study. The elements in plasmid SK+ 157 are described in the section for materials and methods.

- Plasmid +5 SK+ 157

The double-stranded oligonucleotide harbouring five extra bases preceding the original *rbcL* transcription start was cloned into the plasmid SK+ 157. Shown in **Fig. 8.**

The oligonucleotide incorporated a 5'blunt end and a *BspEI* compatible site for cloning. Annealing, phosphorylation of 5' ends using T4 polynucleotide kinase (PNK) and gel purification preceded the cloning steps into plasmid SK+ 157.

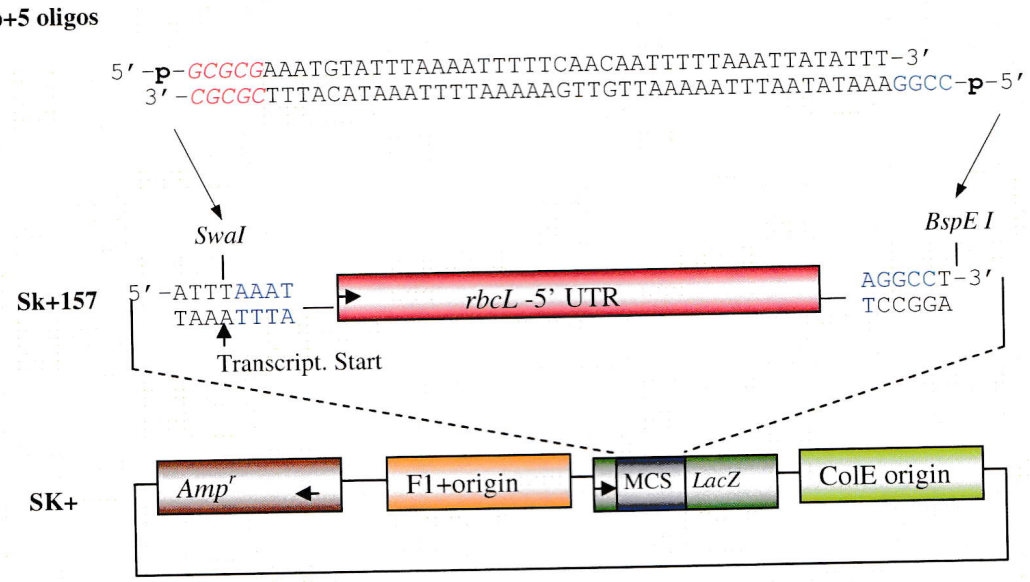


Fig. 8.

Insertion of 5 extra bases in pSK+ 157.

The oligonucleotide is shown above the relevant region of SK+ 157. The extra 5 bases are shown in red. The added phosphates are shown in bold grey (**-p-**). The *rbcL* UTR element is boxed in red. The UTR region incorporates restriction sites used upon digestion of the vector, *SwaI* and *BspEI*.

BspEI compatible sticky-ends and *SwaI* blunt-ends are shown in blue.

More details on construction of plasmid SK+ 157 are given in the materials and methods section.

+5 pSK+ 157 is obtained by ligation of the double strand oligo-fragment in the mentioned digestion sites.

The modified SK+ is about 5.5kb. The arrows indicate direction of transcription of the diverse elements, F1+ and *E.coli* replication origos (F1+ origin and ColE origin), ampicillin resistance (*Amp*^{r}) and *LacZ* gene.

The multiple cloning site is shown in blue (MCS).

- Cloning of *rbcL* 5'UTR construct of plasmid +5 SK+ 157 into the vector MU21

Strategy

Plasmids MU21 and +5 SK+ 157 share many common structural features. It is feasible to use the restriction sites *XhoI* and *XbaI* in order to clone part of the original chimeric construct from the plasmid +5 SK+ 157 into the vector MU21. See **Figs. 6.** and **11.** for details. The 2.2kb *rbcL*/GUS- fragment spanning *rbcL* positions –186 to +126 in plasmid MU21 is exchanged with the 2.1kb *rbcL*/GUS-fragment spanning *rbcL* positions –70 to +162 in +5 SK+ 157.

- Digestions

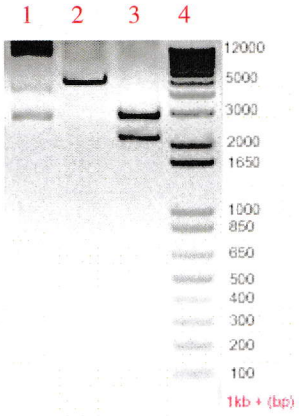


Fig. 9.

Fig. 9. Digestion of the +5-157 *rbcL* UTR construct in plasmid SK+157. Agarose gel electrophoresis 1.

1. Uncut SK+ 157.
   We observe the multiple band pattern of uncut plasmid DNA:
   From bottom to top:
   Nicked, covalently closed and supercoiled DNA migration bands.
2. Single cut linear dsDNA. The plasmid digested with *XhoI* restriction endonuclease has a size of ~5kb.
3. Double digested plasmid with *XhoI/XbaI*.
   The size of the fragments are 3kb and ~2.1kb.
   The lower band belongs to the chimeric *rbcL*/GUS cassette.
4. 1kb + ladder.

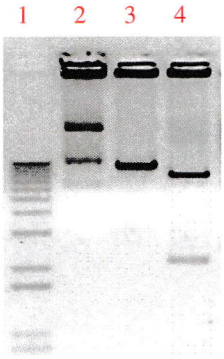


Fig. 10.

Fig. 10. Digestion of the +5-157 *rbcL* UTR construct in plasmid MU21. Agarose gel electrophoresis 2.

1. 1kb + ladder
2. Uncut MU21, with uncut plasmid DNA pattern displayed.
3. Single cut linear plasmid. *XhoI* digestion. The estimated size ~11kb
4. Double digested plasmid with *XhoI/XbaI* restriction enzymes which give 2 bands, a lower band ~2.1kb belonging to the original *rbcL*/GUS cassette in MU21 and ~9kb for the remaining plasmid.

The 2.2kb fragment on gel 1. and the 9kb fragment on gel 2 were isolated, purified by phenol-chloroform extraction and subsequently collected in 70% ice-cold ethanol. Concentration and vector/insert ratios were calculated and ligation performed with T4-DNA ligase following the protocol described in Materials and methods.

E.coli calcium chloride competent cells were transformed with the ligation mix and the cell suspension spread on ampicillin plates and incubated at 37°C for 16h (Sambrook and Russell, 2001).

Minipreparations and colony screening

Transformants were picked up for DNA minipreparation. Four colonies were screened for the presence of the right construct. The transformants harbour the modified version of *rbcL* with the five extra bases insertion. We call the vector plasmid in the transformants for p+5.

The plasmid MU21 harbours the *rbcL* 5' UTR which spans positions –186 to +128 and includes a *SwaI* restriction site which is not present in p+5. **Fig. 11.** The restriction site can be used to distinguish the transformation vector p+5 from its precedent MU21.

Other enzymes wich have expected unique (*Xba I*) or double restriction sites (*Bam HI*) can be used to test the integrity of the p+5 plasmid.

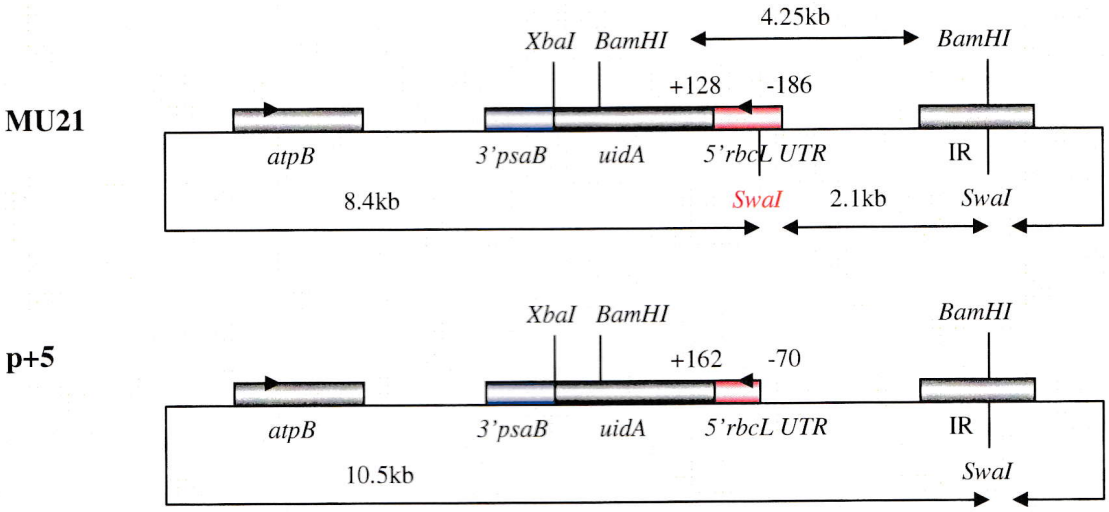


Fig.11.
***Swa I* restriction sites in pMU21 and p+5**

Two representations of the *rbcL*/GUS construct in MU21 and p+5. Both plasmids have a total size of 10.5kb. *Swa I* restriction sites which are used to identify p+5 plasmids in subsequent restriction tests are shown. The disrupted restriction site *Swa I* in MU21 5'UTR is in red. The sizes for the expected DNA fragments obtained after *Swa I* digestion are shown in both cases. Restriction sites *BamHI* and *XbaI* are identical in both plasmids.

Restriction tests

The colonies are analysed for the presence of the *rbcL*/GUS cassette. DNA samples for each colony are digested with appropriate enzymes and loaded on an agarose gel for electrophoresis.

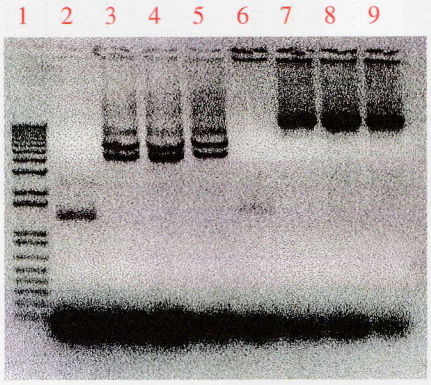


Fig. 12.

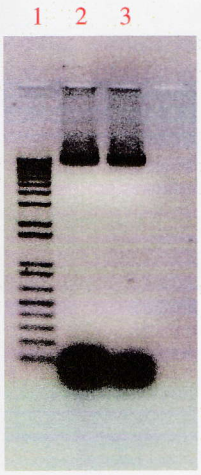


Fig. 13.

Fig. 12.

Four colonies are analysed for the presence of *rbcL*/GUS cassette.

The DNA samples are digested with *Swa I* enzyme.

Nine lanes are shown.

1. The first lane represents 1 kb + ladder

2-5. Uncut plasmids from colonies 1 to 4.

6-9. *Swa I* digested plasmids from colonies 1 to 4.

Swa I gave single-cut digestions as expected in all but one colony (lanes 2 and 6). See **Fig. 11.**

In all cases we obtain linear plasmids with about ~10.5kb size.

Colonies 2 and 4 are taken to further analysis.

Fig. 13.

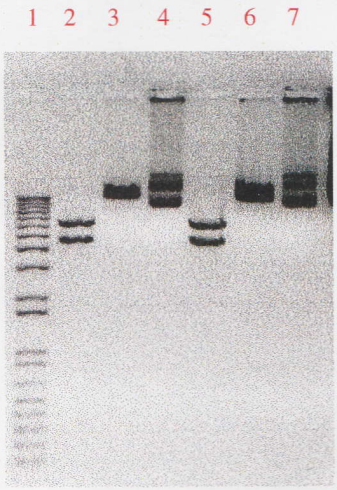
Colonies 2 and 4 are tested with another enzyme with unique restriction sites, *Xba I*.

Three lanes are shown.

1. 1kb ladder.

2-3. In both cases we obtain single cut linear plasmids with about ~10.5kb size.

The dark spots at bottom on both firgures are RNA traces from miniprep.



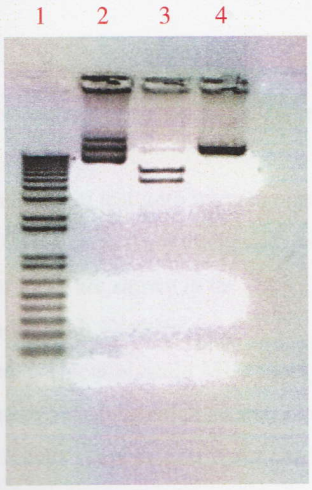


Fig. 14. **Fig. 15.**

Fig. 14.

The previous colonies 2 and 4 are screened again for their plasmids.

Seven lanes are shown in the gel.

1. 1kb + ladder.

2-4. Colony 2. Sequentially: *BamHI* digested, *Swa I* digested, and uncut plasmid.

5-7. Colony 4. Sequentially: *BamHI* digested, *Swa I* digested, and uncut plasmid.

Both Colonies 2. and 4. present the same restriction pattern for *BamHI*.

The lower band is about 4.25kb and belongs to both *rbcL*/GUS construct and part of *Chlamydomonas* IR.

The higher is about 6.25kb and belongs to the rest of the plasmid. See **Fig.11.**

The digestion with *Swa I* gives one single band as in the previous gel.

Fig. 15.

A final test of the transformation vector was performed after a CsCl gradient plasmid maxipreparation.

Four lanes are shown in the gel.

1. 1 kb + ladder.

2. Colony 2. Uncut plasmid

3. *BamHI* digested

4. *Swa I* digested.

The results are identical to the miniprep analysis.

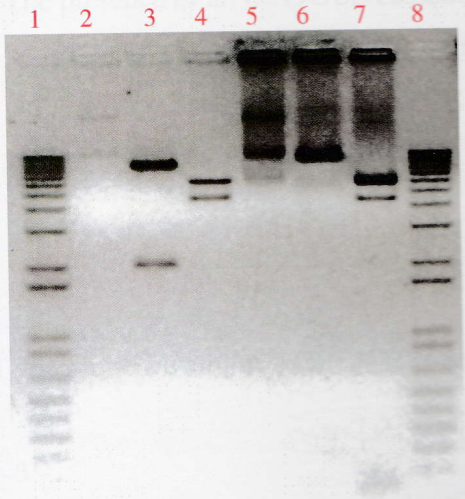


Fig. 16.

Digestion test with *SwaI* and *BamHI*

An additional test with internal negative control for *SwaI* digestion (MU21) is carried out along with p+5 transformation vector.

Eight lanes are shown in the gel.

1 and 8. 1 kb + ladder

2 and 5. Uncut plasmid MU21 and p +5 (Weak signal in MU21).

3 and 6. *Swa I* digestion on MU21 and p +5 respectively.

The results show that the *SwaI* site is present twice in pMU21 and once in p+5. See **Fig.11.**

4 and 7. *BamHI* digestion on MU21 and p+5.

Sequencing

The presence of an *rbcL/GUS* cassette in pMU21 harbouring 5 extra nucleotides was confirmed by means of automatic cycle sequencing by the chain termination method (Sanger et al., 1977). The primer used in PCR is a 21mer that anneals to the GUS region in the plasmid DNA template.

5′ CGCGCTTTCCCACCAACGCTG 3′

The sequence amplified by PCR is depicted below.

5′
CGACTGATAAGACAAGTACATAAATTTGCTAGTTTACATTATTTTTTATTTCTAAATATATA
ATATATTTGCGCGAAATGTATTTAAAATTTTTCAACAATTTTTAAATTATATTTCCGGACAG
ATTATTTTAGGATCGTCAAAAGAAGTTACATTTATTTATATAAATGGTTCCACAAACAGAAA
CTAAAGCAGGTGCTGGATTCAAAGCCGGTGTAAAAGACTACCGTTTAAAATTCCTGCAGCCC
GGGTGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCG
ACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAA
AGCGCG 3′

Fig. 17.
Figure showing plasmid +5 sequence

Colour code
The promotor at position –10 in the *rbcL* UTR region is shown in red.
The 5 extra bases inserted in p+5 are shown in orange
The blue lightened region belongs to the *uidA* (GUS) gene.
The primer complementary region in the GUS sequence is labelled in light green.

2. Analysis of the transformants

After transformation (Biorad Biolistic PDS-1000/He Particle delivery apparatus), each transformant arising from a single colony is grown on HS-agar medium in high light and then transferred to HS liquid media for analysis. Constant illumination is used to select the cell lines harbouring the restored *atpB*+ gene.

The transformants are screened for the presence of *uidA* (GUS) by means of slot blot hybridization and the ratio between integrated *atpB* and GUS genes is assessed with Southern analysis.

Detection of GUS

The slot blot of genomic chloroplast DNA preparations was set up according to the laboratory manual as described in the materials and methods section.

Screening of a total of 8 transformants for the +5 construct is shown in **Fig.18.**

The slot blot membrane was hybridised with a radioactive GUS probe according to the Zeta-probe manual and the results are shown in the following autoradiogram.

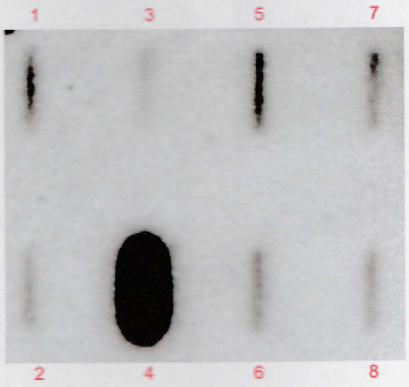


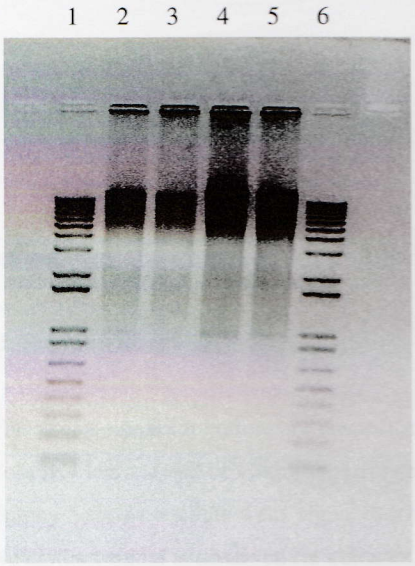
Fig.18.
Slot blot.

The relevant GUS signals with higuest intensity are labelled as Nrs. 4, 5, and 1 on the autoradiogram in decreasing order of intensity. The strongest signals are presumably from the transformants harbouring a high number of genome copies with incorporated +5 *rbcL*/GUS constructs.

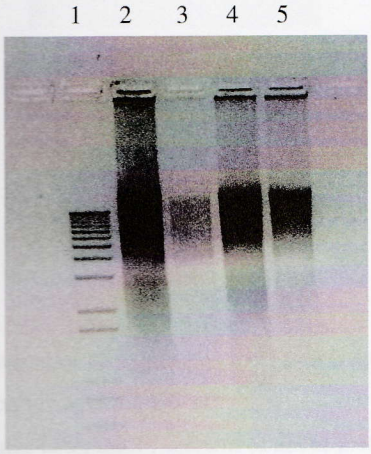
Determining the amount of GUS gene in the transformants

Southern analysis

The Southern technique is used to assess the *rbcL*-GUS/*atpB* ratio.
The transformants in the slot blot with a stronger signal for GUS are taken to Southern analysis. Those belong to colonies 4, 5, 1 and 6. A control (MU7) harbouring an unmodified version of the *rbcL*-GUS construct is also taken along with the colony samples. The DNA samples are digested with *KpnI / HindIII*, loaded in a gel, transferred to a nylon membrane, and hybridised to an *atpB* probe according to the Zeta-probe manual as described in materials and methods.



Gel 1.



Gel 2.

Fig. 19.

Gel 1.
Six lanes are shown.
1. and 6. 1kb ladder.
2-5. *KpnI / HindIII* digested DNA samples of colonies 4, 5, 1 and 6, respectively.

Gel 2.
Five lanes are shown.
1. 1kb ladder.
2-5. correspond to DNA samples of colonies MU7 (Control), 4, 5, and 1 respectively.

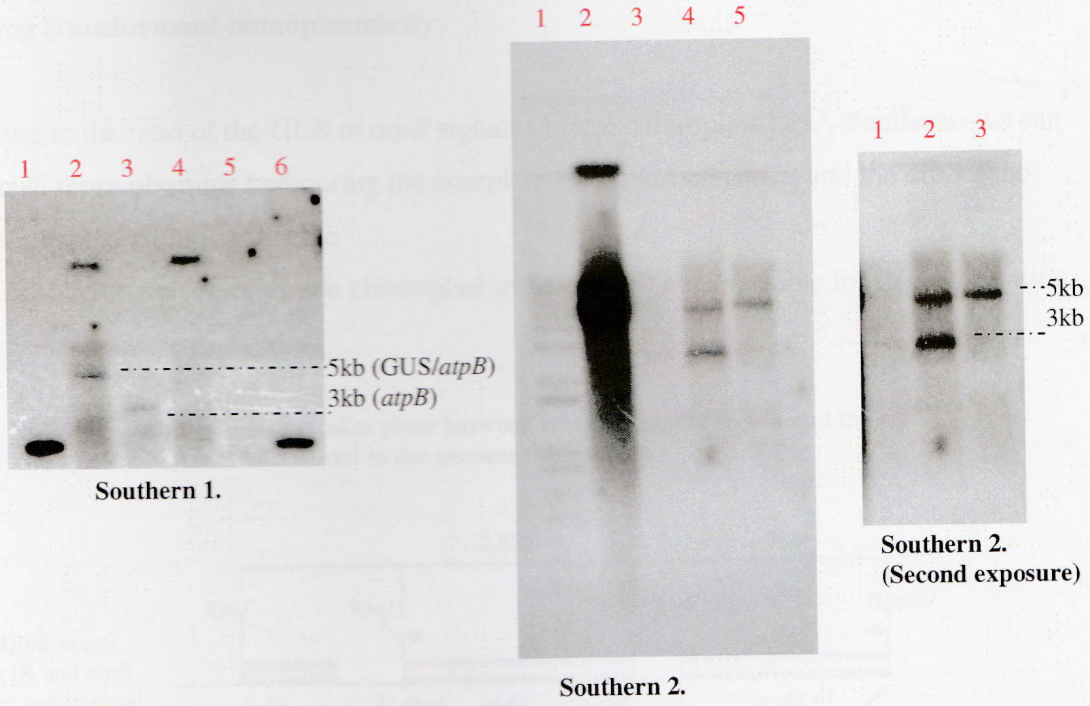


Fig. 20. Autoradiograms

Southern 1. Samples on gel 1.

1. and 6. The strong signals at bottom are unspecific binding of *atpB* probe to the 1.6kb fragment of the 1kb+ ladder.

2. Colony 4 has a signal of ~5kb suggesting the presence of GUS/*atpB*.

3. Colony 5 shows a single weak signal that belongs to an *atpB* fragment of about 2.9kb (no GUS).

4. and 5. No distinct signal is observed for colonies 1 and 6 respectively

Southern 2. Samples on gel 2.

1. and 2. Unspecific binding of *atpB* probe to the 1kb ladder and MU7 control respectively.
The stronger signal in the smearing for MU7 on 5kb region suggests the presence of GUS/*atpB*.

3. Colony 1 shows a weak band of ~2.9kb belonging to the *atpB* fragment (no GUS).

4. Colony number 5 shows both 5 and 2.9kb bands.
This suggests a more heterogeneous distribution of *rbcL*/GUS.

5. Colony number 4 has a signal of ~5kb suggesting the presence of the GUS gene.

Southern 2. (Second exposure)

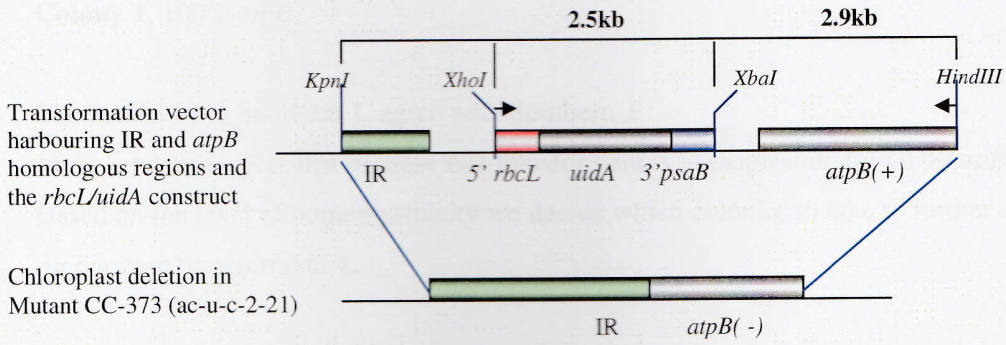
1-3. Two days exposition of the membrane to the X ray film increases the signal intensities of the bands.
Only signals for colonies 1, 5, and 4 are shown.

Assessing transformant homoplasmicity

According to the ratio of the GUS to *atpB* signals in total chloroplast DNA-Southerns we can distinguish those plasmids harbouring the complete *rbcL/uidA* construct and the *atpB* gene from those with just the *atpB* gene.

The recombination processes upon chloroplast transformation responsible for this distribution are depicted in the figure below.

1. **Homologous recombination takes place between inverted repeat region and the *atpB* (+) gene. The whole cassette is introduced in the genome (blue lines).**



2. **Homologous recombination takes place between flanking sequences in *atpB* (+). Just the *atpB* gene is inserted in the genome (blue lines).**

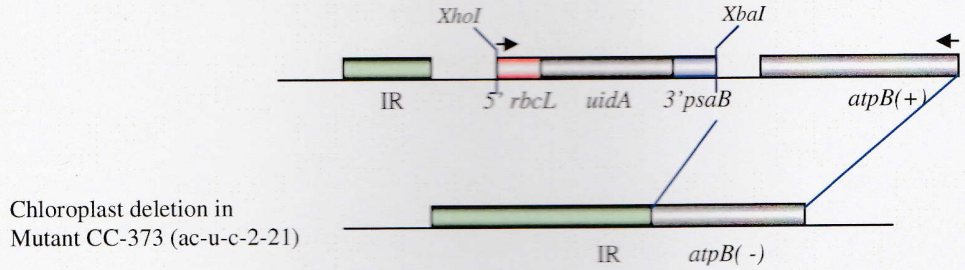


Fig. 21.

Possible recombination processes in *Chlamydomonas* chloroplast transformants. The two alternatives for homologous recombination of *rbcL/uidA* constructs are shown in pictures 1. and 2.

In homoplasmic transformants, every copy in the genome has the complete cassette.
The level of homoplasmicity can be estimated by comparing the intensities of the 5kb and 3kb bands on DNA gel Southern blots

Levels of homoplasmicity of the transformants

See Southern 2. (2^{nd}exposure) and Southern 1. for assessment of levels of homoplasmicity in **Fig. 20.** Judging from the results of Southern 2. the levels of homoplasmicity are

Colony 4. 100% GUS/*atpB*
Colony 5. 40% GUS/*atpB*, 60% *atpB*
Colony 1. 100% *atpB*

The results from Southern 1. agree with Southern 2.
MU7 is taken as a control because this transformant is homoplasmic for GUS/*atpB*.
Based on the level of homoplasmicity we decide which colonies to take to further analysis, in our case transformant 4.

Estimating the stability of the construct

Northern analysis was used to measure levels of GUS expression in transformants. Samples of RNA in dark or light where collected, loaded in a gel and hybridised to the GUS probe according to the Zeta-probe manual as described in materials and methods.

Northern analysis

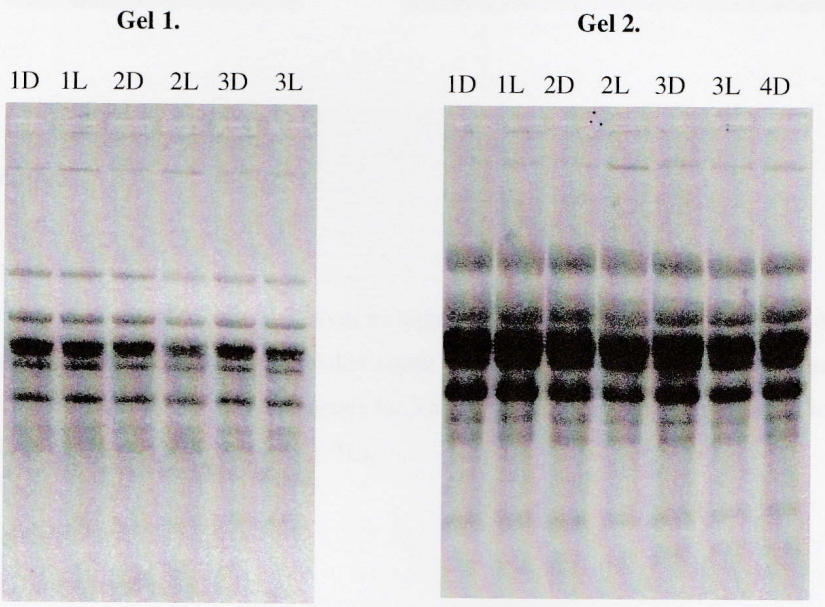


Fig. 22.

Gels. *Chlamydomonas* chloroplast RNA samples

Gel 1.

A typical pattern of ribosomal RNA fragments is seen in every lane. D. Dark samples, L. Light samples.

1 D/L transformant 4

2 D/L and 3 D/L MU7 (control)

Gel 2.

D. Dark samples, L. Light samples.

1 D/L transformant 4

2 D/L transformant 5

3 D/L transformant 1

4 D MU7 (control)

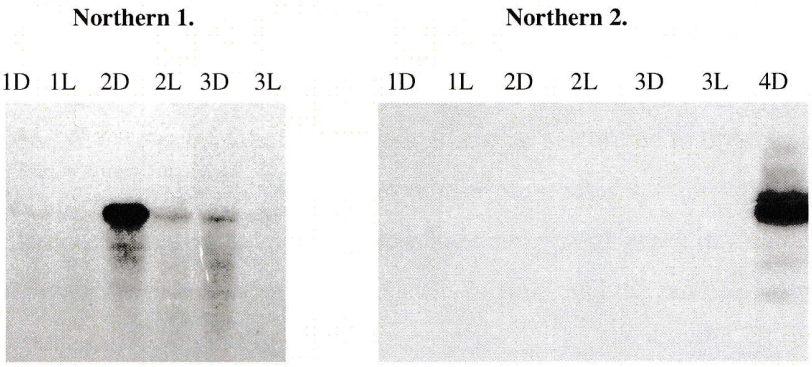


Fig. 23.
Autoradiograms

Northern 1. Samples in gel 1.
RNA from Colony 4, samples (1D, 1L), gives no signal neither in light nor in the dark .The MU7 control has a strong signal in the dark samples and a weaker signal in the light samples (2D versus 2L) and (3D versus 3L). The difference in intensity between the signals for 3 and 2 are due to variable amounts of loaded MU7 RNA (4 μg on every sample versus < 2μg in 3D, 3L).

Northern 2. Samples in gel 2.
RNA from colonies 4, 5, and 1 give no signal neither in light nor dark samples (1-3 D, 1-3 L). This is consistent with the results in Northern 1. The strong signal on 4D belongs to control MU7.

The results of both Northerns show that there is no accumulation of *rbcL*/GUS transcript for any of the +5 transformants neither in light nor in the dark samples while control MU7 show variable amounts of *rbcL*/GUS transcripts in both light and dark samples suggesting that the additional 5 nucleotides at the transcripts 5' terminus destabilize the RNA.
MU7 is a control transformant that harbors the chimeric cassette and includes *rbcL* 5'UTR region (positions –290 to +97 relative to transcription start). The 5'UTR harbours the basal promotor element and downstream stabilizing elements from positions +1 to +93.

In vivo determination of transcription rates.

The Northerns show that RNA transcripts for construct +5 are not present neither in light or dark samples. In vivo pulse labeling analysis has to be performed in order to elucidate if this is due to transcript destabilization or lower transcription rates.
GUS +5 chimeric construct is compared to other endogenous genes in *Chlamydomonas*. Transcript levels are measured at short intervals of time, and the radioactive pulses are shown in the autoradiogram below. **Fig. 24.**

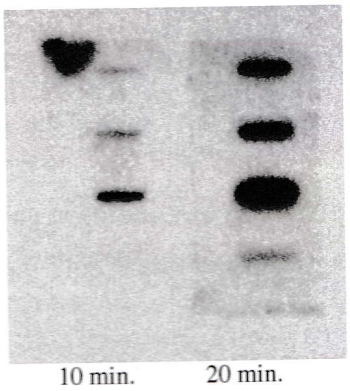


Fig.24. In-vivo pulse labeling.

Measurement of transcription rates for *rbcL* in +5 construct, *atpB* and *rbcL* endogenous genes. pUC18 is a control plasmid. Measurements were taken at 10 and 20 min. intervals respectively ***(Salvador et al., 2004)***.

The results indicate that the chimeric gene transcript is indeed transcribed actively.
The signals of *rbcL*/GUS in +5 are close to the signals of the endogenous *atpB* transcripts at both 10min and 20min intervals. Endogenous *rbcL* transcripts have stronger signals than *rbcL* transcripts for the +5 construct in both intervals of time presumably reflecting the instability of *rbcL* +5 transcripts in the assay.

IV. Discussion

1. Effects of the 5 basepair insertion

The 5bp insertion has a surprising effect on transcript accumulation. The half life of *rbcL*/GUS transcripts drops drastically from 4 to 5 hours in the control *rbcL*/GUS transformant to barely 5 min in constructs in which the stem structure is modified (Salvador et al., 2004). This suggests that the extra 5 nucleotides at the 5' terminus of the *rbcL* UTR decrease the longevity of the transcripts by at least 50 fold.

The effect of the 5bp insertion on 5'UTR secondary structure is depicted in **Fig. 25.**
The stability element spanning positions +38 to +47 (relative to transcription start) basepairs differently in the +5 construct. This is accompanied by a global change in secondary structure.

Transcript stability could require the intervention of nuclear factors or cpRNPs that increase the longevity of the transcripts (Nickelsen, 2003). A model involving transcript stabilization mediated by cpRNPs has been suggested (Nickelsen, 2003).
This may also be the case for *rbcL* transcripts in *Chlamydomonas* (Salvador et al., 2004)

Low levels of transcript accumulation do not correlate with changes in transcription rate as shown in the in-vivo pulse labeling of RNA transcripts in **Fig. 24.**
There was litle difference between transcription rate in endogenous *rbcL* gene and the +5 construct. This points towards transcript unstabilization as the principal effect of the 5bp insertion construct.

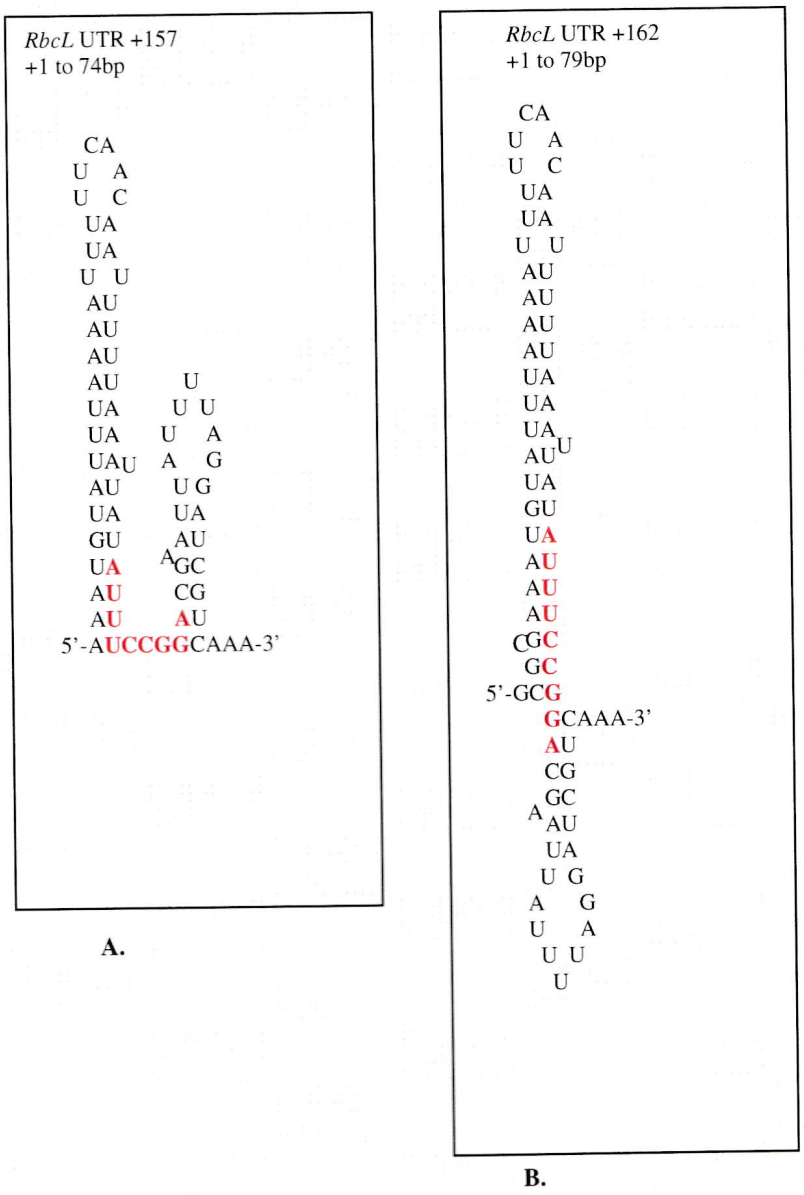


Fig.25.

Predicted secondary structures of *rbcL* 5'UTRs.

A. Unmodified transcript, wild type, spanning positions +1 to +74bp.

B. 5bp insertion construct, +1 to +79bp.

The sequence elements having a demonstrated effect in transcript stability are highlighted in bold red (Anthonisen et al., 1995). Note how the base pairing within the stability element from +38 to +47 bp has changed in the right figure, resulting in a totally different fold of the transcript in this region.

Based on computer simulation using the Mfold program (Zuker et al., 1999).

2. Mechanisms involved in chloroplast mRNA stability

RNA-binding proteins such as endo-exonucleases seem to be temporary or permanent components of RNA-binding complexes involved in mRNA stability (Nickelsen, 2003). The possible candidates are

- Translation complexes
- Degradation complexes (endonucleases)
- 5'-3' end-processing complexes
- Intron processing complexes
- Editing complexes

2.1 Translation mediated transcript stabilization model (Nickelsen, 2003)

Chloroplast RNA-binding proteins (cpRNPs), might have revealed an alternative function in mRNA degradation by binding to cognate elements in RNA 5'UTRs.
Two cpRNPs, Nac2 and Mbb1, control the stabilisation of *psbD* and *psbB* gene transcripts respectively (Boudreaux et al., 2000; Vaistij et al., 2000; Auchincloss et al., 2002).

A figure illustrating the model for the *psbD* gene is shown in **Fig. 26.**
In the *psbD* gene, Nac2 protein interacts directly or by means of other proteins (complex) with a cognate RNA element upon transcription, the PRB2 element (Nickelsen et al., 1999). This confers protection to the RNA against exonuclease-mediated transcript degradation in 5' to 3' direction. cpRNPs can also function as a scaffold for other catalytic machineries involved in RNA processing or translation.
RBP-40 is a cpRNP protein mediating translation initiation. Nac2 guides RBP-40 to an U-rich binding site immediately downstream of PRB2 (Ossenbühl and Nickelsen, 2000).
Afterwards, Nac2 protein releases from the transcript and RBP-40 mediated ribosome assembly takes place.

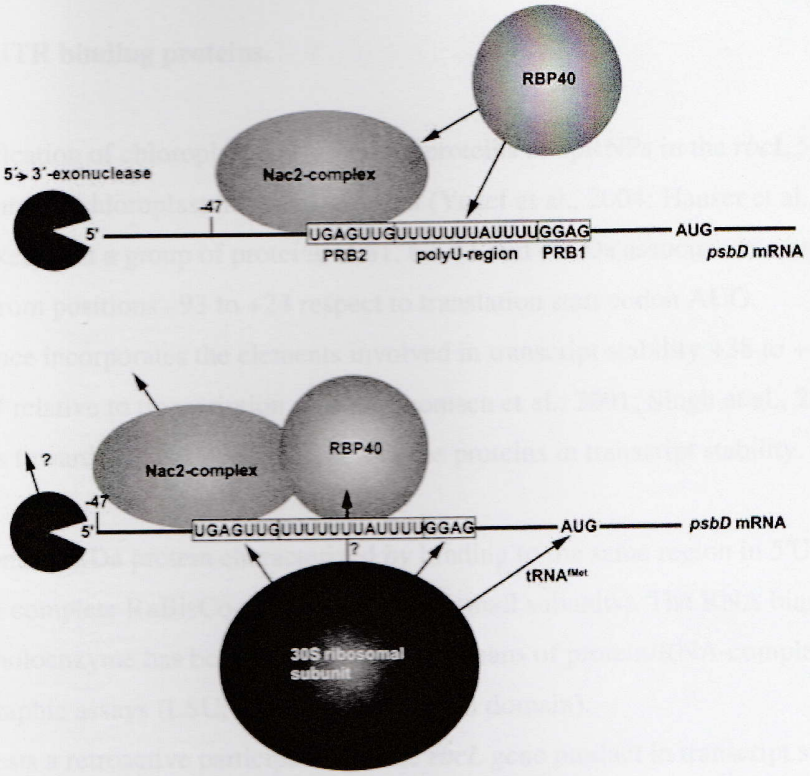


Fig.26.

Translation mediated transcript stabilization.

RNA stabilisation depends basically on both the presence and effective interaction of Nac2 complex on the RNA and links transcript stabilisation to the translation initiation processes.
It is likely that Nac2 complex association inhibits a 5'-3' exonuclease.

- Step 1. Top.

Nac2 protein-complex interacts with a RNA element and the protein factor PRB2 within the 5'UTR in *psbD* gene (Nickelsen et al., 1999). This leads to protection of the RNA against 5'-3' exorybonuclease.

- Step 2. Bottom.

Nac2 interacts with a secondary trans-acting factor RBP40 which binds to Nac2-complex and recognizes a poly-U stretch in the 5'UTR (Ossenbühl and Nickelsen, 2000).
Once RBP40-RNA complex has been established, Nac2 protein is released (arrow on Nac2-complex) and 30S ribosomal subunit recognizes direct or indirectly RBP40 complex sponsoring translation (arrows towards transcription start codon, AUG and elements in the sequence). It is unclear how the exonuclease recognition of the transcript is prevented (arrow with question mark).

2. *rbcL* 5'UTR binding proteins.

The identification of chloroplast RNA binding proteins or cpRNPs in the *rbcL* 5'UTR of *Chlamydomonas* chloroplast has been reported (Yosef et al., 2004; Hauser et al., 1996)
It seems likely that a group of proteins of 81, 62, 51 and 47kDa associate to a sequence spanning from positions –93 to +24 respect to translation start codon AUG.
The sequence incorporates the elements involved in transcript stability +38 to +47 and +14 to +27 relative to transcription start (Anthonisen et al., 2001; Singh et al., 2001).
This points towards a possible implication of the proteins in transcript stability.

An additional 55kDa protein characterized by binding to the same region in 5'UTR in *rbcL* gene is the complete RuBisCo protein (large and small subunits). The RNA binding site of RuBisCo holoenzyme has been characterized by means of protein/RNA-complex crystallographic assays (LSU, large subunit protein domain).
This suggests a retroactive participation of the *rbcL* gene product in transcript stabilization and more generally in post-transcriptional processes of gene expression (Yosef et al., 2004).

In addition, a general mechanism for the function of the mentioned proteins binding the 5'UTR in *rbcL* gene has been proposed. It is likely that all proteins identified but RuBisCo are sensitive to redox state and dissociate in oxidative conditions (e.g) Bis-[Gamma-glutamyl-L cysteinyl glycine]-disulfide, (GSSG). The interaction of the proteins with their cognate elements in the UTR seems to be specific. It is unclear if the nature of the interaction depends on the sequence or the secondary structure (Yosef et al., 2004).

Some of the proteins have been characterized in relation to other elements in *psbA* gene.
(e.g) 5'UTR binding proteins +47 and +60kDa (Danon et al., 1991).
The +47kDa protein has been identified as RB47, a poly-A Binding protein.
The +60kDa protein has been identified as RB60, a disulfide isomerase that is probably a redox sensor for +47 protein.

RuBisCo does recognize the 5'UTR leader in *rbcL* gene although the nature of the interaction has shown to be less specific.

RuBisCo protein associates to *rbcL* transcript upon oxidative-stress conditions and hence independently of the other mentioned proteins (Yosef et al., 2004).

RuBisCo's binding to the *rbcL* in 5'UTR could correlate with transcript stabilization since the stabilization of *rbcL* transcripts in light is improved by oxidative conditions (Salvador et al., 1993a).

It would be exciting to confirm if this is indeed the case for *rbcL* and/or other genes like the chloroplast *atpB* gene.

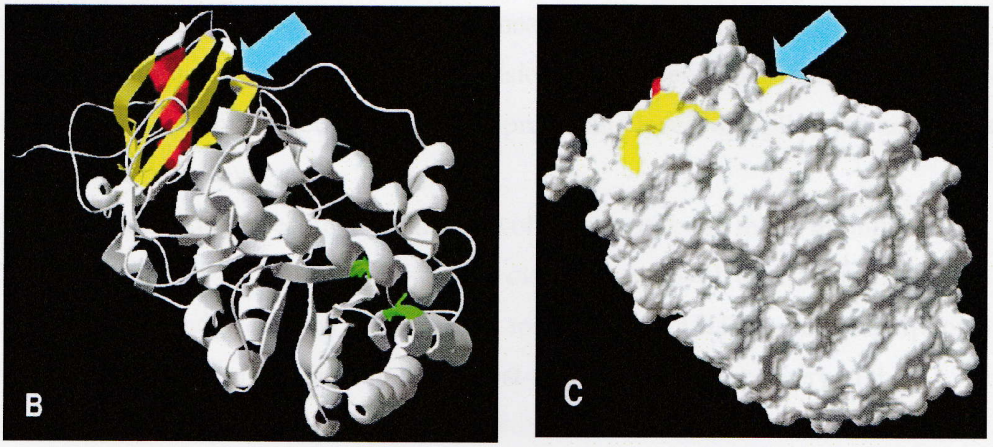


Fig. 27.

B.

The crystal structure of Rubisco large subunit highlighting the RNA binding domain, blue arrow.

C.

Surface representation of Rubisco large subunit emphasizing the inaccessibility of RNA under reducing conditions. Oxidizing conditions initiate a conformational change in Rubisco, which exposes the RNA binding domain.

The RNA binding domain consists of a N-terminal domain (residues 10-87).

β strands in yellow and α-helix in red are highlighted. This domain has a strong homology with the human RNA binding-domain of the splicing factor, U1A.

(Yosef et al., 2004)

V. Conclusion and perspectives

The understanding of the communication system between chloroplast and nucleus will give answers to fundamental questions in biology concerning how an unicellular photoautotrophic eukaryote evolved by starting from two independent organisms, a photoautotrophic cyanobacterium and a heterotrophic eukaryote.

Furthermore, the advantages that the *Chlamydomonas* chloroplast offers upon transformation and its structural and functional similarity with higher-plant chloroplasts combined by the extensive molecular work done to date may allow a solid understanding of post-transcriptional processes in the near-future.

The concrete study of cis-element functions by means of in-vivo structural studies and transcription analyses will give a more precise vision of the interactions and requirements of the transcript sequence and structure at different processing levels as well as the nature of the regulation mechanisms involved.

This work has studied the effect that the addition of 5 nucleotides to the 5' terminus of the *rbcL* 5'UTR has in establishing a function of the cis-elements involved in transcriptional RNA stability. The study confirms the important role that the 5'UTR has in transcriptional stability and extends our understanding of the post-transcriptional machinery in the chloroplast.

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