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**Manipulation of Gene Expression in the Chloroplast:
Control mRNA Stability and Transcription Termination**

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Control mRNA stability and transcription termination**

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Abstract

Chloroplasts are the site of photosynthesis and of other essential biosynthetic activities in plant cells. Chloroplasts are semi-autonomous organelles, since they contain their own genomes and protein biosynthetic machinery, but depend on the coordinate expression of nuclear genes to assemble macromolecular complexes. The bioengineering of plants requires manipulation of chloroplast gene expression, and thus a knowledge of the molecular mechanisms that modulate mRNA and protein production.

In this proposal the heterotrophic green alga *Chlamydomonas reinhardtii* has been used as a model system to understand the control and interrelationships between transcription termination, mRNA 3' end processing and mRNA stability in chloroplasts. *Chlamydomonas* is a unique and ideal system in which to address these issues, because the chloroplast can be easily manipulated by genetic transformation techniques. This research uncovered new and important information on chloroplast mRNA 3' end formation and mRNA stability. In particular, the 3' untranslated regions of chloroplast mRNAs were shown not to be efficient transcription terminators. The endonucleolytic site in the 3' untranslated region was characterized by site directed mutagenesis and the role of several 3' untranslated regions in modulating RNA stability and translation has been studied. This information will allow us to experimentally manipulate the expression of chloroplast genes *in vivo* by post-transcriptional mechanisms, and should be widely applicable to other higher plant systems.

2. Objectives of the original research proposal

Our original objectives in the funded proposal were:

1. To determine the specificity of an *atpB* 3' end processing endonuclease by site-directed mutagenesis and *in vitro* RNA processing;
2. To purify this endonuclease and isolate the gene that encodes it;
3. To measure transcription termination at different 3' sequences *in vivo*;
4. To extend our *in vitro* RNA processing results *in vivo* by using chloroplast transformation techniques; and
5. To study the processing pathways of additional mRNA 3' ends.

3. Results

Our experimental objectives and results from the proposal are as follows:

1. *in vitro* analysis of the *atpB* endonuclease cleavage site.

The precise nature of endonuclease cleavage in the *atpB* 3' UTR suggests that this activity may recognize specific sequences and/or RNA structures. As a first step to address this possibility, several site-directed mutants were constructed and analyzed in the *in vitro* processing system. Two examples, illustrating the negative and positive results obtained are presented in Figure 1. When four nucleotides were replaced at the endonuclease cleavage site (ECS) (construct MUT.2, *Nsi* in figure 2), there was no change in the processing pattern that could be detected by the resolution of this experiment. However, when the site was more drastically changed to include only G and C residues, the cleavage site "moved" about 40-50 nt downstream of the previous site (construct MUT.1, *not* in figure 2). Surprisingly, there was no accumulation of the mature product. Therefore the exonuclease, under these *in vitro* conditions, did not stop at the inverted repeat and generate a correct product, but instead continued resulting in degradation of the RNA. The low amount of product that could be detected is probably from pausing of the exonuclease in the stem-loop region. These results suggest that like the bacterial endonuclease RNaseE, when the endonuclease cleavage site is blocked, cryptic site(s) may be used (1). However, the location of the "normal" site as related to the stem-loop, may be important for the generation of a stable and mature 3' end processing product. The site directed mutants that were constructed are presented in Figure 2. They were tested by the *in vitro* processing assay as described in Figure 1. In addition these mutants were introduced by genetic transformation into *Chlamydomonas* cells. The analysis of these transformants is in progress.

2. Purification and characterization of the endonuclease.

Fractionation the processing activity on anion exchange and affinity media indicated that the endonuclease activity did not bind to a heparin column, but did bind to the anion exchange resourceQ FPLC column (Figure 3). Nevertheless, in the progress of the purification procedure we faced the problems that the endonuclease activity was masked by a strong exonuclease one and/or was inactivated. These obstacles seems to be solved recently and therefore additional trial will be performed to biochemically isolate this enzyme.

Figure 1. Mutation in the endonuclease cleavage site can alter *in vitro* 3' end processing. A) Schematic presentation of the *atpB* 3' end sequences and the two site directed mutants that were generated to alter the nucleotide sequences in the endonuclease cleavage site. B) Chloroplast protein extract was incubated with *in vitro* transcribed RNA corresponding to the 3' end of the *atpB* gene and the mutants shown in A. RNA was isolated at each time point and analyzed on denaturing polyacrylamide gel and autoradiography. The sizes of the mature 3' end processing product, the endonuclease cleavage product (endo.) and the product accumulated with MUT.1 RNA (mut. Prod.), are shown to the right of the figure.

<u>P17-IR ENDO Mutants</u>	
	<div>3' end</div> <div>Endo. site</div>
wt	CATACATTATATATATGTCATATTAGCTATTTAATCTATTTACTTT
not	GCGGCCGC --
Sma	CCCGGG --
Sal	GTCGAC --
Sal fi	GTCGATC GAC
Nsi	GATGCA --
Nsi fi	G ATTAGC (GATTAGC)

Figure 2: The site directed mutants generated to study the endonuclease specificity.

Figure 3 Fractionation of chloroplast extract on resource Q and heparin columns. **A)** Chloroplast protein extract was fractionated on Heparin agarose affinity column. The flow-through (Hep. FT) and the bound proteins that were eluted with 1 M KCl (Hep. B) were analyzed in in vitro 3' end RNA processing assay. RNA was isolated after 5, 30 and 60 min of incubation and analyzed on denatured polyacrylamide gel and autoradiography. **B)** Fractionation of the chloroplast protein extract on resource Q anion exchange column. Fractions were analyzed in the *atpB* in vitro 3' end RNA processing assay. The incubation time was 60 min. The lanes denote (-) and T represent RNA incubated without proteins and with total chloroplast protein, respectively. The numbers indicate the fractions of the resourceQ column.

3. Transcription termination.

When the 3' UTR of the *atpB* gene was analyzed in run-on transcription experiments, it was found that more than 50% of the 3' ends of *atpB* mRNA are generated by processing of precursor RNAs (pre-mRNAs) rather than by transcription termination (2). To address the question of whether the 3' UTRs of other *Chlamydomonas* chloroplast genes are also inefficient transcription terminators, the 3' UTRs of the *petD*, *petD* plus *trnR* and *petA* genes were placed between the *petD* promoter and the *uidA* or *aadA* reporter genes (Figure 4; (3)). The *trnR* gene lies immediately downstream of *petD* and was included in some constructs because *petD* 3' end processing may depend on RNase P cleavage at the 5' end of *trnR*. Since the *E. coli* threonine-attenuator is an efficient terminator in higher plant chloroplasts (4), we tested to determine if this is also the case in *Chlamydomonas* chloroplasts. To determine if termination efficiency is orientation-dependent, each 3' UTR was introduced in both sense and antisense orientations (Figure 4A). We used two different reporter genes in order to exclude the possibility that the results of the run on experiments would have been effected by some properties of the reporter gene. These and other chimeric genes were inserted in the large inverted repeat, downstream of *atpB* and in opposite orientation (Figure 4B). The resulting recombinant plasmids were used to transform the nonphotosynthetic recipient strain CC373 which lacks a functional *atpB* gene (Figure 4B). Phototrophic transformants were selected and analyzed for correct integration and homoplasmy by DNA filter hybridization.

Figure 4 **A)** Schematic representation of the constructs used to analyze transcription termination in the *chlamydomonas* chloroplast. A 3' untranslated region of a chloroplast gene, shown schematically as stem loop structure, was inserted in sense (I) or antisense (II) orientations between a promoter and a reporter gene. The amount of the reporter-gene transcript produced in a run on experiment was used to estimate the efficiency of the 3' UTR to function as a transcriptional terminator. **B)** Map of the chloroplast genome *atpB* region with the termination constructs shown as insert. The region deleted in the recipient strain cc373 is indicated by open box. Selected restriction enzyme recognition sites are as follows: B, BamHI; S, SphI; X, XhoI; E, EcoRI. IR - indicates the inverted repeat of the chloroplast genome. The directions of the coding region of the genes is shown by arrows. The different 3' end untranslated regions of the chloroplast genes that were inserted in the SphI site are shown schematically as a stem and loop structure.

To determine transcription termination efficiency, RNA was pulsed-labeled in permeabilized cells, and hybridized with filter-bound RNA corresponding to the reporter gene antisense strand. The amount of the radioactive reporter gene mRNA produced in the run-on assay was quantified by normalization to the hybridization signal of the *petB* gene. Results of several such experiments with transformed *Chlamydomonas* cells are summarized in Figure 5 and shown in the manuscript in which this set of experiments was described (3). It is evident from these data that the 3' UTRs tested do not serve as efficient transcriptional terminators, and therefore that the 3' ends of most or all chloroplast mRNAs are probably generated by processing of pre-mRNAs. This result emphasizes the importance of accurate and efficient *in vivo* processing mechanisms in chloroplasts.

Table I: Plasmid constructs used for *Chlamydomonas* chloroplast transformation

Plasmid	Promoter	3' UTR (orientation)	Length of insert (bp)	Reporter Gene
1. pDG2	<i>petD</i>	-	-	<i>uidA</i>
2. pDAAD	"	-	-	<i>aadA</i>
3. GD +	"	<i>petD</i> (+)	320	<i>uidA</i>
4. GD -	"	<i>petD</i> (-)	"	<i>uidA</i>
5. AD +	"	<i>petD</i> (+)	"	<i>aadA</i>
6. AD-	"	<i>petD</i> (-)	"	<i>aadA</i>
7. GDt+	"	<i>petD</i> + <i>trnR</i> (+)	538	<i>uidA</i>
8. GDt -	"	<i>petD</i> + <i>trnR</i> (-)	"	<i>uidA</i>
9. ADt+	"	<i>petD</i> + <i>trnR</i> (+)	"	<i>aadA</i>
10. ADt -	"	<i>petD</i> + <i>trnR</i> (-)	"	<i>aadA</i>
11. AA+	"	<i>petA</i> (+)	254	<i>aadA</i>
12. AA-	"	<i>petA</i> (-)	"	<i>aadA</i>
13. GR+	"	<i>rbcL</i> (+)	437	<i>uidA</i>
14. GT+	"	<i>E. coli</i> , threonine attenuator (+)	227	<i>uidA</i>

Fig. 5. Relative transcription termination activity of the different 3' UTRs. The relative transcriptional activities of the test sequences shown in the Table were calculated by normalizing the amounts of hybridized labeled run-on transcripts to the reporter gene probes to that of the *petB* gene, using data as shown in Fig. 5. The left ordinate shows readthrough of the reporter gene sequence, with results from the strain lacking a test sequence (DG2 or DAAD) set to 100%. The right ordinate shows the inferred termination percentage, with 100% read-through of the test sequence being interpreted as 0% termination. Mean values and experimental errors are shown, with two to three independent assays being performed for each strain. ND = not done.

4. *in vivo* analysis of IR function and endonuclease processing.

Endonuclease cleavage in the *atpB* 3' UTR leads to both *atpB* mRNA maturation and also to rapid degradation of downstream sequences (5). To determine the sequence requirements for these functions *in vivo*, and also to set the stage for a genetic strategy to isolate ribonuclease mutants, we used a translational fusion of the *Chlamydomonas petD* promoter and 5' (UTR) fused to the accumulates a stable *uidA* transcript and expresses GUS activity (6). A series of plasmids was constructed in which 200-300 bp derivatives of the *atpB* 3' IR and endonuclease cleavage site (ECS) were inserted into the BglII site at position +25 of the *petD* 5' UTR, as shown in Figure 6A. These plasmids each contain a functional *atpB* gene for selection of chloroplast transformants. The plasmids were designated Ω 1DG, Ω 2DG, Ω 4DG, Ω 8DG and Ω 21DG (Fig. 6B). Plasmid Ω 1DG contains the ECS but not the IR region, Ω 2DG contains both the IR and the ECS in the sense orientation, Ω 4DG contains both the IR and the ECS in the antisense orientation, Ω 8DG contains both the IR and the ECS in the normal orientation followed by the 3' IR of the spinach *petD* gene and strain Ω 21DG contains the *atpB* 3' IR but not the ECS. Another set of plasmids was constructed in which the *uidA* coding region was replaced by the coding region of the *aadA* gene. These plasmids were designated DAAD, Ω 1DA, Ω 2DA and Ω 4DA, respectively. Transplastomic *Chlamydomonas* strains containing the modified *uidA* or *aadA* gene cassettes were isolated after biolistic transformation of the nonphotosynthetic *Chlamydomonas* strain CC373, as described above.

Figure 6 The Ω DG *petD-uidA-rbcL* fusion cassettes and accumulated *uidA* transcripts of the Ω DG *Chlamydomonas* strains.

A) The structure of the *petD-uidA-rbcL* fusion cassette of *Chlamydomonas* strain DG2 showing the BglII site at position +25. The broken horizontal line denotes that functional *atpB* gene is not drawn to scale. The arrows above the genes indicate the direction of transcription. **B)** The structure of the *atpB* 3' regions which have been inserted into the BglII site of the DG2 fusion, creating the Ω DG constructs. The stem-loop structures represent inverted repeats and the vertical arrows represent the endonuclease cleavage site of *Chlamydomonas atpB* 3' region. The dashed stem-loop of Ω 8DG is derived from the spinach chloroplast *petD* 3' region. **C)** Accumulated *uidA* transcript of *Chlamydomonas* strains DG2, P17 (wild-type), Ω 1DG, Ω 2DG, Ω 4DG, Ω 8DG and Ω 21DG. 10 μ g of total RNA from each strain was fractionated in a denaturing agarose gel and transferred to a nylon membrane. The membrane was hybridized with double-stranded DNA probe derived from the *uidA* coding region. The sizes of the accumulated transcripts are shown at right. The dashed arrow designates higher molecular weight transcripts which may represent read-through from functional *atpB* gene.

The accumulated *uidA* transcripts of *Chlamydomonas* strains P17 (wild-type), DG2, Ω 1DG, Ω 2DG, Ω 4DG, Ω 8DG and Ω 21DG were examined by RNA filter hybridization; the results are shown in Figure 6C. A 1.9 kb *uidA* transcript accumulates in DG2, and *uidA* transcripts of approximately 2.1-2.2 kb accumulate in strains Ω 4DG and Ω 21DG, and weakly in Ω 1DG. Strains Ω 2DG and Ω 8DG do not accumulate *uidA* transcripts of this size. In some of the strains the *uidA* probe hybridizes with a larger transcript which may represent read-through antisense transcripts emanating from the functional *atpB* gene. Because Ω 1DG, Ω 2DG and Ω 8DG encode transcripts that can be cleaved by an endonuclease *in vitro*, the RNA filter hybridization results are consistent with the working hypothesis that cleavage at the *atpB* ECS causes destabilization of the downstream cleavage product, in this case the *uidA* transcript. Hybridization with a second chloroplast probe (*petD*) was used to confirm equal loading of the different RNAs (data not shown). Therefore, a system has been established in which instability of a reporter gene transcript appears to be correlated with an endonucleolytic cleavage event that requires the sense strand of the *atpB* 3' ECS. The destabilization is not specific to the *uidA* reporter gene, because similar results were obtained with transformants carrying *aadA* fusion cassettes. An alternative interpretation of these results is that transcription terminates only at the Ω 2 and Ω 8 sequences, and the *uidA* transcript is not synthesized in these strains. Although this was unlikely because these sequences have been shown to be inefficient transcription terminators *in vivo*, transcriptional run-off analysis was performed to show that the *uidA* transcription rate is unaffected by the insertions (data not shown).

RNA and antibiotic resistance analysis was carried out for the *aadA*-fusion plasmids described above. DAAD, which has no insertion in the *petD* 5' UTR, was resistant to 400 μ g/ml spectinomycin (data not shown). Ω 1DA was resistant to 100 mg/ml spectinomycin, but sensitive to 200 mg/ml and Ω 4DA was resistant to 200 μ g/ml spectinomycin, but sensitive to 400 μ g/ml spectinomycin (Table II). RNA filter hybridization analysis revealed a strong *aadA*-hybridizing signal in DAAD, but no transcript was visible for Ω 1DA and only a very weak signal was seen for Ω 4DA (Table II). This is consistent with the Ω 1 sequence causing destabilization of the *aadA* transcript as it does for *uidA*, however it is not clear why the Ω 4DA transcript accumulates to lower levels than that of DAAD. It is also remarkable that Ω 1DA cells are resistant to low levels of spectinomycin, however it should be noted that even when the *aadA* product is immunologically undetectable drug resistance can still be conferred to *Chlamydomonas* cells.

Table II: RNA and antibiotic analysis of the *aadA*- fusion transformants (For details see the text)

	<i>aadA</i> RNA	spectinomycin	antibiotics
		resistant ($\mu\text{g/ml}$)	sensitive ($\mu\text{g/ml}$)
DAAD	+++	400	
Ω 1DA	-	100	200
Ω 4DA	- +	200	400

5. The sequence and structure of the 3' untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability

To examine 3' untranslated region (UTR) function *in vivo*, the *atpB* 3' UTR was replaced with the 3' UTR sequences of the *Chlamydomonas* chloroplast genes *petD*, *petD* plus *trnR*, *rbcL*, *petA* and *E. coli thrA* by biolistic transformation of *Chlamydomonas* chloroplasts (Figure 7). Each 3' UTR was inserted in both the sense and antisense orientations. The accumulation of both total *atpB* mRNA and ATPase β -subunit protein was approximately equal to that of wild type in all of the transformants (Figure 8; (7)). However, the level of discrete *atpB* transcripts in transformants containing the antisense 3' UTR sequences was reduced to approximately one-half that of transformants containing the 3' UTRs in the sense orientation (Figure 9). These results imply that both the nucleotide sequences and the stem-loop structures of the 3' UTRs are important for transcript 3' end processing, and accumulation of the mature mRNAs. It also suggests that in these transformants, the level of correctly processed mRNA does not limit protein accumulation.

Figure 7: The *Chlamydomonas* chloroplast *atpB* region.

Map of the 7.6 kb BamHI fragment of the

Chlamydomonas chloroplast genome. A portion of the large inverted repeat of the chloroplast genome is shown as an open arrow. The inverted repeat of the *atpB* 3' UTR is shown as a stem-loop structure. The extents of the deletions in the chloroplast genomes of strains CC373 and Δ 26 are indicated.

Table III: *Chlamydomonas* chloroplast *atpB* transformants

Transformant	3' UTR (orientation)	Length of insert in bp
$\Delta 26$	none (N/A)	none
D+	<i>petD</i> (sense)	320
D-	<i>petD</i> (antisense)	320
Dt+	<i>petD-trnR</i> (sense)	538
Dt-	<i>petD-trnR</i> (antisense)	538
A+	<i>petA</i> (sense)	254
A-	<i>petA</i> (antisense)	254
R+	<i>rbcL</i> (sense)	437
R-	<i>rbcL</i> (antisense)	437
T+	<i>E. coli thrA</i> (sense)	227

Figure 8: Accumulation of the ATPase β -subunit.

A. Total proteins from the indicated transformants were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated sequentially with antibodies directed against the ATPase β -subunit (top panel) and the D1 protein of photosystem II (lower panel). Antigenic proteins were visualized by chemiluminescence.

B. Densitometric quantification of autoradiograms of the immunoblots such as those shown in Figure 5A. Accumulation of the ATPase

β -subunit was normalized to D1 protein, and displayed as a fraction of the accumulation in wild-type cells.

Figure 9: Accumulation of transcripts from chimeric *atpB* genes.

Total RNA (15 μ g) from the indicated transformants was fractionated in an agarose gel, transferred to a nylon membrane and hybridized sequentially with 32 P-labeled *atpB* and *psbA* probes.

The mechanisms of chloroplast mRNA 3' end formation have been unraveled largely through the use of *in vitro* RNA processing systems (4) (8) (2,9,10) (11). We have extended the previous analysis of *Chlamydomonas* chloroplast *atpB* mRNA 3' end formation by showing that 3' UTR of the *petD* can also be processed *in vitro*. However, processing of the *petA* 3' UTR transcript, which does not contain IR sequences, did not result in accumulation of a product (7), even though a *petA* transcript of discrete size accumulates *in vivo* (3). It is likely that either factor(s) required for transcript processing are missing from the chloroplast extract, or that the substrate provided does not form the requisite structure. Antisense *atpB* 3' UTR RNA, which can form a stem-loop structure similar to that of the sense strand, was degraded without the accumulation of a stable product. These results demonstrate that a stem-loop structure is required, but not sufficient for mRNA 3' end processing *in vitro*.

Our studies also address a second issue, which is the importance of 3' end processing in chloroplast gene expression. Nucleus-encoded mRNAs, for example, must undergo 3' cleavage and polyadenylation in order to reach the cytosol and be efficiently translated reviewed in (12). Elaborate mechanisms have also evolved to process the cell cycle-regulated histone mRNAs at their 3' stem-loop. Here using chloroplast transformation, we have tested whether the 3' UTRs of the *Chlamydomonas reinhardtii* chloroplast *petD*, *petA* and *rbcL* genes, or the *E. coli* threonine attenuator, can substitute for the wild-type *atpB* 3' UTR *in vivo*. Compared to a transformant lacking the *atpB* 3' IR ($\Delta 26$), transformants containing *atpB* genes flanked by any of the 3' UTRs in either the sense or antisense orientation, accumulated increased levels of *atpB* transcripts and ATPase β -subunit. However, there were significant differences in the accumulation of discrete *atpB* transcripts between transformants harboring the 3' UTRs in sense or antisense orientations. Transcripts of *atpB* genes flanked by the sense orientations of the 3' UTRs tested accumulated almost entirely as homogeneous populations of discrete RNA molecules. Although the accumulation of total *atpB* transcripts flanked by the antisense orientations of the 3' UTRs was similar to that of transcripts flanked by the sense orientations, the proportion of transcripts which accumulate as discrete RNA molecules was reduced by approximately one-half. The remainder, which was detected by slot-blot analysis,

could either be shorter (and untranslatable) or longer than the discrete molecules. The exception to this observation was antisense orientation of the *petD-trnR* sequences, which resulted in accumulation of the wild-type level of discrete *atpB* transcript. The reason for this is not clear, but it is possible that the antisense strand fortuitously contains efficient processing signals.

Since the chimeric *atpB* genes are identical except for the 3' UTR sequences, we interpret our results as evidence that the antisense 3' UTRs are less efficient at forming 3' ends resistant to exonuclease degradation. Although the antisense UTRs (except *petA*) contain inverted repeats that can form stem-loop structures, the nucleotide sequences of the loop and regions flanking the stem are different. These results, as well as those of Blowers *et al.* (13), are consistent with the idea that the loop and/or IR flanking sequences influence the efficiency of 3' end processing. For example, they may contain binding site(s) for proteins and/or nucleases that perform the 3' end processing of the transcript. Both *Chlamydomonas atpB* and spinach *petD* 3' end processing are initiated by specific endonucleolytic cleavages just downstream of the mature 3' end (5) (11). Comparisons of the nucleotide sequences of several *Chlamydomonas* 3' UTRs did not reveal a consensus sequence downstream of the inverted repeat, although few mature 3' ends have been mapped precisely in *Chlamydomonas* chloroplasts. We note, however, that the sequence UGUCAU, which is the site of endonucleolytic cleavage of the *atpB* 3' primary transcript, is also found 10 nucleotides downstream of the mature 3' end of *petD* 3' mRNA (7). *In vitro* analysis of *atpB* transcripts containing mutations in this sequence demonstrated that in some cases, 3' end processing was affected (R. Rott and G. Schuster, unpublished). Therefore, the lack of specific endonuclease target site sequences could be one reason for the inability of the antisense sequences to direct efficient 3' end processing of the *atpB* transcript.

While strain $\Delta 26$, which lacks the *atpB* 3' IR, accumulates only 30% of the wild-type level of the ATPase β -subunit, the transformants harboring the experimental 3' UTRs accumulated wild-type levels of protein. This suggests that for these strains, translatable RNA is not limiting for protein accumulation. Because the chloroplast ATPase complex is quite stable (14), there are several possible explanations for this observation. One possibility is that only transcripts with correctly processed 3' ends are translatable, and 30% of the wild-type level of processed *atpB* transcript is sufficient to support translation and accumulation of wild-type protein levels. Alternatively, 3' end processing is not required for translation, and transformants exceeding some critical threshold of *atpB* transcripts containing the entire coding region can support sufficient protein synthesis to accumulate the wild-type level of protein. Another possibility is that there are compensating mechanisms in the chloroplast which increase the rate of translation or decrease the rate of protein degradation, resulting in the accumulation of the wild-type level of protein. Association of unspliced and unprocessed transcripts with translating polysomes has been reported in maize chloroplasts (15), and analysis of the interaction of *Chlamydomonas atpB* transcripts containing the inserted 3' UTRs with polysomes may address this issue.

Cooperation

The Principal Investigator visited the co-PI's laboratory in the Technion and the co-PI visited the PI's laboratory twice. In addition, a graduate student from the Technion spent a month in Ithaca working on aspects of this proposal in the PI's laboratory. Different constructs and strains have been sent back and forth between Israel and the U.S., with chloroplast transformation being carried out in the U.S., and some of the cloning and *in vitro*

work being carried out in Israel. This close collaboration is best reflected in the joint publications of the two laboratories: two in 1996, one in 1997 and several in preparation.

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6. List of Publications

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