

Complementarity of the 16S rRNA penultimate stem with sequences downstream of the AUG destabilizes the plastid mRNAs

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ABSTRACT

Escherichia coli mRNA translation is facilitated by sequences upstream and downstream of the initiation codon, called Shine–Dalgarno (SD) and downstream box (DB) sequences, respectively. In *E. coli* enhancing the complementarity between the DB sequences and the 16S rRNA penultimate stem resulted in increased protein accumulation without a significant affect on mRNA stability. The objective of this study was to test whether enhancing the complementarity of plastid mRNAs downstream of the AUG (downstream sequence or DS) with the 16S rRNA penultimate stem (anti-DS or ADS region) enhances protein accumulation. The test system was the tobacco plastid rRNA operon promoter fused with the *E. coli* phage T7 gene 10 (T7g10) 5'-untranslated region (5'-UTR) and DB region. Translation efficiency was tested by measuring neomycin phosphotransferase (NPTII) accumulation in tobacco chloroplasts. We report here that the phage T7g10 5'-UTR and DB region promotes accumulation of NPTII up to ~16% of total soluble leaf protein (TSP). Enhanced mRNA stability and an improved NPTII yield (~23% of TSP) was obtained from a construct in which the T7g10 5'-UTR was linked with the NPTII coding region via a *NheI* site. However, replacing the T7g10 DB region with the plastid DS sequence reduced NPTII and mRNA levels to 0.16 and 28%, respectively. Reduced NPTII accumulation is in part due to accelerated mRNA turnover.

INTRODUCTION

In *Escherichia coli* translation is facilitated by mRNA–rRNA interactions between the Shine–Dalgarno (SD) sequence upstream of the AUG translation initiation codon and the anti-Shine–Dalgarno sequence (ASD) at the 3'-end of the small (16S) rRNA. A second mRNA element is the downstream box (DB). DB is defined as a 15 nt sequence downstream of the AUG translational initiation codon complementary to the penultimate stem of the 16S rRNA or the anti-downstream box (ADB) (nt 1469–1483) (1,2). In *E. coli* increasing complementarity of the

DB sequence to the 16S rRNA ADB region resulted in a significant increase in protein accumulation (3,4).

In higher plant plastids mRNA sequences in the 5'-untranslated region (5'-UTR) were shown to be important for translation. These sequences are complementary to the 16S rRNA 3'-end and may be SD-like (GGA) (5) or distinct from SD, such as RBS1 (AAG) and RBS2 (UGAUGAU) (6). Signals for light-dependent mRNA translation (7,8) and mRNA stability (9) are also localized in the 5'-UTR. The sequence context around the translation initiation codon is conserved (10) and is probably important for efficient translation. Silent mutations downstream of the *rbcL* AUG dramatically reduced (35-fold) translation efficiency without an effect on mRNA stability (11). Since both the 5'-UTR and sequences downstream of AUG affect protein accumulation they will collectively be referred to as the translation control region (TCR). Sequences enhancing translation downstream of the AUG in plastids have been termed downstream sequences (DS) to distinguish them from the *E. coli* DB sequence (11). The role of the 5'-UTR in regulating translation and mRNA stability is best characterized in the chloroplasts of the unicellular alga *Chlamydomonas* (12–14). General rules deduced from the *Chlamydomonas* studies may accelerate the understanding of translational regulation in higher plants.

To explore the feasibility of using heterologous translation control signals in combination with plastid promoters we have fused the strong σ^{70} -type plastid rRNA operon promoter (Prnn) with the T7 phage gene 10 (T7g10) TCR. The T7g10 TCR was shown to promote high level protein accumulation in bacteria (15) and has a well-characterized DB sequence (2). Specifically, we tested if increasing complementarity between the plastid 16S rRNA penultimate stem (ADS) and sequences downstream of the AUG (DS sequence) facilitates enhanced neomycin phosphotransferase (NPTII) accumulation.

We report here that the phage T7g10 TCR promotes accumulation of NPTII up to ~16% of total soluble leaf protein (TSP). Thus, the heterologous phage TCR, when fused to a strong plastid promoter, is suitable for high level expression of recombinant proteins in chloroplasts. Enhanced mRNA stability and an improved NPTII yield (~23% of TSP) was obtained from a construct in which the T7g10 5'-UTR was linked with the NPTII coding region via a *NheI* site. However, replacing the T7g10 DB region with the cognate plastid DS

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sequence dramatically reduced NPTII accumulation (to ~1% of the T7g10+DB/*Ec* control) and accelerated mRNA turnover.

MATERIALS AND METHODS

Plasmid construction

Prrn is the σ^{70} -type promoter of the plastid rRNA operon (16,17). The chimeric Prrn promoters are contained in *SacI*-*NheI* fragments in pBSIIKS⁺ derivatives. The PrrnLT7g10+DB/*Ec* cassette (Prrn promoter with the T7g10 leader and *E.coli* DB) is carried by plasmid pHK18. The PrrnLT7g10+DS/pt cassette (Prrn promoter with the T7g10 leader and plastid DS) is carried by plasmid pHK19. The PrrnLT7g10 cassette (Prrn promoter with the T7g10 leader and *NheI* site) is carried by plasmid pHK20. The promoter fragments were constructed by PCR. Construction details are available upon request.

In the pHK plasmids listed above the *neo* coding region from plasmid pSC1 (18) is expressed in a cassette consisting of the Prrn promoter fragment and TrbcL (the plastid *rbcl* gene 3'-UTR) (19). The Prrn promoter derivative and the *neo* coding region are linked via the engineered *NheI* site. Plastid vectors pHK38, pHK39 and pHK40 were obtained by cloning the *neo* gene from plasmids pHK18, pHK19 and pHK20 as *SacI*-*HindIII* fragments into plastid vector pPRV111A (20).

Plastid transformation and regeneration of transgenic plants

Transforming DNA (Plasmid Maxi Kit; Qiagen) was introduced into leaf chloroplasts on the surface of tungsten particles (1 μ m) using the Du Pont PDS1000He Biolistic gun. Transplastomic plants were selected on RMOP medium containing 500 mg/l spectinomycin dihydrochloride. A uniform population of transformed plastid genome copies was confirmed by DNA gel blot analysis (17). The transgenic plants were grown on MS (Murashige-Skoog) medium (21) containing 3% sucrose and 0.6% agar under sterile culture conditions.

RNA gel blot analysis

Total cellular RNA was prepared from the leaves of plants grown in sterile culture (22). The RNA (4 μ g/lane) was electrophoresed on 1.2% agarose/formaldehyde gels and then transferred to Hybond N membranes (Amersham) using the PosiBlot Transfer apparatus (Stratagene). Hybridization to the probe was carried out in Rapid Hybridization Buffer (Amersham) overnight at 65°C. Double-stranded DNA probe was prepared by random primed ³²P-labeling. The template for probing *neo* was a gel purified *NheI*-*XbaI* fragment excised from plasmid pHK30. The template for probing the tobacco cytoplasmic 25S rRNA was a PCR fragment amplified from total tobacco cellular DNA with primers 5'-TCACCTGCCGAATCAACTAGC-3' and 5'-GACTTCCCTTGCCTACATTG-3'. RNA hybridization signals were quantified using a Molecular Dynamics PhosphorImager and normalized to the 25S rRNA signal.

SDS-PAGE and immunoblot analysis

Leaves for protein extraction were taken from plants grown in sterile culture. To obtain TSP, leaves (200 mg) from plants grown in sterile culture were homogenized in 1 ml buffer containing 50 mM HEPES-KOH pH 7.5, 10 mM potassium

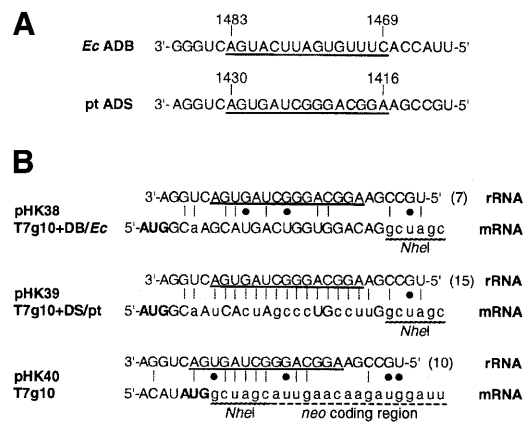


Figure 1. The *E.coli* mRNA DB and plastid mRNA DS regions. (A) The 15 nt ADB regions in the *E.coli* (*Ec*) 16S rRNA (nt 1469–1483) (2,45) and the cognate DS region in the tobacco plastid (pt) 16S rRNA (nt 1416 and 1430) (46). (B) Complementarity of the mRNA DB and DS regions with the plastid 16S rRNA penultimate stem ADS region. Watson–Crick (lines) and G-U (filled circles) pairs are marked. Nucleotides corresponding to the *NheI* site and *neo* coding region are in lower case. The numbers of potential pairs formed with the 15 nt DS region are in parentheses.

acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol and 2 mM phenylmethylsulfonyl flouride. The homogenate was centrifuged to remove the insoluble material. Protein concentrations were determined with a Bio-Rad Protein Assay reagent kit (Bradford). The proteins were separated by SDS–PAGE (15% acrylamide, 6 M urea) (23), followed by either staining with Coomassie Brilliant Blue R250 or immunoblotting. For immunoblot analysis protein was transferred onto nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad). After blocking, the membrane was incubated with 4000-fold diluted polyclonal rabbit antiserum raised against NPTII (5Prime→3Prime Inc.). 20 000-fold diluted HRP-conjugated secondary antibody and ECL chemiluminescence reagent (Amersham) were used for immunoblot detection on X-ray film (Kodak). NPTII was quantified on the immunoblots by densitometric analysis with the DensoSpot program of Alpha Imager 2000 (Alpha Innotech) by comparison of the experimental samples with a dilution series of commercial NPTII (5Prime→3Prime Inc.).

RESULTS

Experimental design

In *E.coli* the DB was defined as a 15 nt sequence downstream of the AUG translational initiation codon complementary to the penultimate stem (ADB region) of the 16S rRNA. The penultimate stem–loop structure of the 16S rRNA is conserved between *E.coli* and plastids, although the actual mRNA sequence is different (Fig. 1; 2,24). The chimeric genes have the same promoter (Prrn), 5'-UTR (T7g10), coding region (*neo*) and 3'-UTR (TrbcL) and differ only with respect to the *neo* coding region N-terminus. The design of the three chimeric promoter/TCR constructs is as follows. In plasmid pHK38 the Prrn derivative has the phage T7g10 5'-UTR and DB (Figs 1B and 2A). In plasmid pHK39 the Prrn promoter derivative has the phage T7g10 5'-UTR and the plastid DS

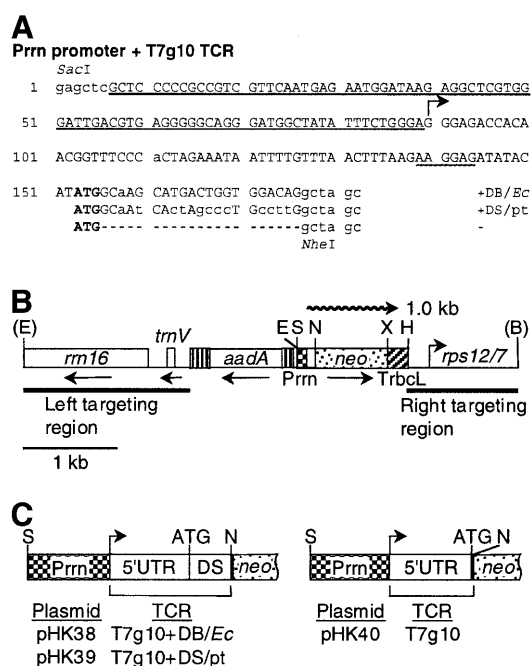


Figure 2. Plasmid vectors to compare translation efficiency from *neo* mRNAs with the *E. coli* DB and plastid DS regions. (A) DNA sequence of Prm promoters with T7g10 leader derivatives. The Prm promoter region is underlined; the transcription initiation site is marked by a horizontal arrow; the translation initiation codon (ATG) is in bold; the SD sequence is underlined with a wavy line. The T7g10 DB (*Ec*) sequence is shown on top; the sequence with the plastid DS region (pt) is duplicated in the middle; the sequence with the *NheI* site is at the bottom. Plasmid and T7g10 sequences are in capital letters; nucleotides added or modified during construction are in lowercase; gaps are marked with dashes. (B) The plasmid targeting region of the transformation vectors. The spectinomycin resistance (*aadA*) and *neo* genes and plastid genes *rrn16*, *tmv* and *rps12/7* (46) are shown. The positions of the *neo* promoter (Prm) and 3'-UTR (TrbcL) are also marked. The wavy line represents 1.0 kb *neo* mRNA. Abbreviations for restriction sites: E, *EcoRI*; S, *SacI*; N, *NheI*; X, *XbaI*; H, *HindIII*; B, *BglIII*. Restriction sites removed during plasmid construction are in parentheses. (C) Listing of plasmids and the schematic map of their promoter and N-terminal coding regions. For an explanation see (B).

sequence. The plastid DS sequence is defined as the sequence complementary to the tobacco plastid 16S rRNA penultimate stem corresponding to the *E. coli* ADB region (ADS sequence;

T7g10+DB/Ec	AUG	GCa	AGC	AUG	ACU	GGU	GGA	CAG	gcu	agc	auu	gaa	caa	gau
	Met	Ala	Ser	Met	Thr	Gly	Gly	Gln	Ala	Ser	Ile	Glu	Gln	Asp
Fraction	1.00	0.29	0.07	1.00	0.37	0.38	0.24	0.43	0.39	0.07	0.45	0.62	0.57	0.75
Triplet/1000	24.6	18.1	4.7	24.6	18.4	28.2	17.9	15.5	24.4	4.7	25.9	20.7	21.0	24.6
T7g10+DS/pt	AUG	GCa	AuC	Acu	Agc	ccU	Gcc	uuG	gcu	agc	auu	gaa	caa	gau
	Met	Ala	Ile	Thr	Ser	Pro	Ala	Leu	Ala	Ser	Ile	Glu	Gln	Asp
Fraction	1.00	0.29	0.27	0.37	0.07	0.30	0.16	0.24	0.39	0.07	0.45	0.62	0.57	0.75
Triplet/1000	24.6	18.1	15.5	18.4	4.7	13.5	10.1	35.7	24.4	4.7	25.9	20.7	21.0	24.6
T7g10	AUG	gcu	agc	auu	gaa	caa	gau	gga	uug	cac	gca	ggu	ucu	ccg
	Met	Ala	Ser	Ile	Glu	Gln	Asp	Gly	Leu	His	Ala	Gly	Ser	Pro
Fraction	1.00	0.39	0.07	0.45	0.62	0.57	0.75	0.24	0.24	0.28	0.29	0.38	0.31	0.30
Triplet/1000	24.6	24.4	4.7	25.9	20.7	21.0	24.6	17.9	34.7	9.1	18.1	28.2	20.2	13.2

Figure 3. Codon frequency in the *neo* coding region N-terminus. Values are shown for the Prm derivatives T7g10+DB/Ec (plasmid pHK38), T7g10+DS/pt (plasmid pHK39) and T7g10 (plasmid pHK40). Fraction is the relative usage frequency of a specific codon compared to the usage frequency of all codons encoding the same amino acid. Triplet/1000 is the frequency of occurrence of a certain triplet per 1000 triplets.

Figs 1B and 2A). In plasmid pHK40 the Prm promoter is fused with the phage T7g10 5'-UTR and the leader is linked to the *neo* coding region via a *NheI* site (Figs 1B and 2A).

Replacement of frequently used codons with rare codons (1.4/2.1 per 1000 codons) in *E. coli* mRNAs results in reduced protein accumulation (25,26). To avoid the potential adverse effects of introducing rare codons into the *neo* coding region, codon usage frequencies were calculated for the chimeric genes. None of the changes made involved replacing a frequently used codon with a codon that would be considered rare by the *E. coli* criteria (≤ 4.6 per 1000 codons; Fig. 3). Codon usage frequencies shown in Figure 3 were calculated for the highly expressed photosynthetic genes *rbcL*, *psaA*, *psaB*, *psaC*, *psbA*, *psbB*, *psbC*, *psbD*, *psbE* and *psbF* (27).

In the plastid transformation vectors the *neo* genes are physically linked with the selectable marker *aadA* and are flanked by plastid DNA to target insertion of *aadA* and *neo* into the *trnV/rps12/7* intergenic region (Fig. 2B). A schematic map of the promoter regions is depicted in Figure 2C. The *neo* genes were introduced into the tobacco plastid genome by standard protocols and the leaves of transplastomic tobacco plants were tested for NPTII accumulation.

NPTII accumulation in plastids

NPTII was quantified by immunoblot analysis or staining of gels with Coomassie Brilliant Blue (Fig. 4A and B). The data are summarized in Table 1. Surprisingly, NPTII from the heterologous T7g10 5'-UTR (DB/*Ec*, Nt-pHK38) and the T7g10 leader combined with the *NheI* site (Nt-pHK40) accumulated to very high levels, ~16 and ~23% of TSP, respectively. These expression levels are comparable to the most highly expressed chloroplast proteins such as the Rubisco large and small subunits (Fig. 4B). This result indicates that highly efficient translation of NPTII from the *neo* mRNA occurred with the heterologous T7g10 5'-UTR combined with the T7g10 DB (DB/*Ec*) and the *NheI* site.

Based on the *E. coli* studies (2–4), we expected that incorporation of the plastid DS would further enhance NPTII levels. However, we found that plants transformed with the construct having the perfect plastid DS (pHK39) contained NPTII levels 100-fold lower than plants expressing NPTII from the T7g10 TCR (pHK38). This result indicates that, unlike in *E. coli* (2,4),

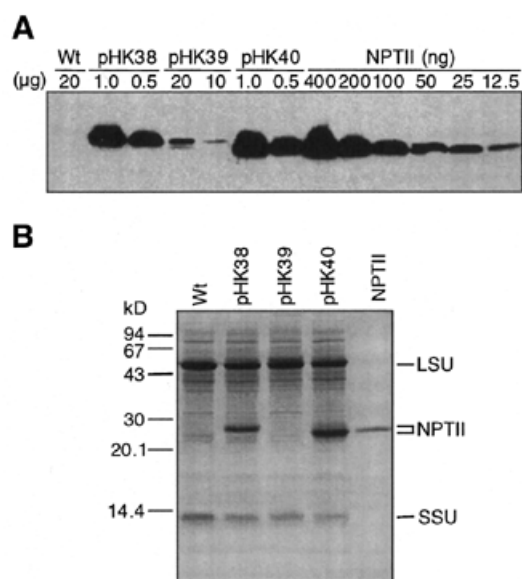


Figure 4. NPTII accumulation in tobacco leaves. **(A)** Immunoblot analysis to detect NPTII. The amount of TSP (μg) loaded on the SDS-PAGE gel is indicated above the lane. A NPTII dilution series was electrophoresed on the same gel. Lanes are marked with the plasmid name used for plant transformation. A protein sample from wild-type tobacco was also loaded (Wt). **(B)** Protein gel ($20 \mu\text{g}/\text{lane}$) stained with Coomassie Brilliant Blue R250. Control, NPTII (700 ng). The positions of NPTII and the Rubisco large (LSU) and small (SSU) subunits are marked.

100% complementarity between the mRNA DS and 16S rRNA ADS region reduces protein accumulation in chloroplasts.

Table 1. Levels of NPTII and *neo* mRNA

Plant line ^a	DB/DS ^b	<i>neo</i> mRNA ^{c,d}	NPTII (%) ^{d,e}
Nt-pHK38-2E	<i>Ec</i>	52.72 ± 21.11	16.39 ± 3.42
Nt-pHK39-3B	pt	14.53 ± 1.41	0.16 ± 0.13
Nt-pHK40-12B	–	100.00	23.00 ± 5.40

^aPlasmid name (for example pHK38), number and letter combination identify plant lines.

^b*Ec*, *E. coli*; pt, tobacco plastid; –, absent.

^cPercent of level in Nt-pHK40 plants.

^dAverage of three to six measurements.

^ePercent of total soluble cellular protein.

Perfect DS–ADS complementarity destabilizes plastid mRNAs

Steady-state mRNA levels also affect protein levels, as more stable mRNAs are available for translation for longer periods of time. Since the three *neo* genes are transcribed from the same promoter (Prnn), differences in steady-state mRNA levels directly reflect differences in mRNA turnover rates. Therefore, RNA gel blot analysis was carried out to determine steady-state *neo* mRNA levels in the transplastomic lines. Quantification of the RNA blots revealed significant differences in *neo* mRNA accumulation (Fig. 5 and Table 1). Highest levels were found in the Nt-pHK40 plants (T7g10 leader, *NheI* site), which were

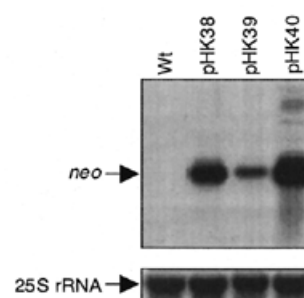


Figure 5. Steady-state levels of *neo* mRNA in the transplastomic leaves. An aliquot of $4 \mu\text{g}$ total cellular RNA was loaded per lane. The blots were probed for *neo* (top) and cytoplasmic 25S rRNA (bottom, loading control). The lanes are designated as in Figure 3.

then used as the reference (100%). The *neo* mRNA levels were half as much ($\sim 50\%$) in plants with the T7g10 DB (Nt-pHK38) and ~ 7 -fold less ($\sim 14\%$) in plants with the perfect plastid DS (Nt-pHK39). Thus, incorporation of a *NheI* site slightly enhanced mRNA stability (Nt-pHK40 plants) while the perfect plastid DS downstream of the AUG significantly accelerated *neo* mRNA turnover (Nt-pHK39 plants).

DISCUSSION

We report here that increasing complementarity between sequences downstream of the AUG with the 16S rRNA 3'-region will reduce, rather than enhance, protein accumulation in chloroplasts. The chimeric *neo* genes are transcribed from the same promoter in the transplastomic lines. Thus, differences in the mRNA levels in the tobacco lines in part are due to differential mRNA turnover. While increased complementarity between the mRNA DS region and the 16S rRNA ADS region destabilized the *neo* mRNA in tobacco chloroplasts, it had no effect on mRNA stability in *E. coli* (2,4). There may be multiple alternative mechanisms that are the cause of accelerated mRNA turnover in chloroplasts. An obvious possibility would be formation of a stable mRNA–rRNA complex that interferes with translational elongation and ultimately triggers mRNA degradation. However, at least in *E. coli*, direct interaction between the mRNA DB box and 16S rRNA ADB sequence is unlikely (28). A more likely scenario is accelerated turnover via the general mRNA degradation pathway. RNA degradation in chloroplasts is initiated by an endonucleolytic cleavage followed by polyadenylation and degradation. The majority of the cleavage sites form in the coding region and near the start of the 3'-UTR (29,30). In an alternative mechanism, the endonucleolytic cleavage is followed by a 5'→3' exonuclease activity (31,32). Furthermore, conformational changes of the mRNA may expose known cleavage sites in the 5'-UTR involved in regulating mRNA turnover in response to light and developmental signals (33,34).

Replacement of DB with DS sequences in the *neo* constructs resulted in an ~ 4 -fold reduction in mRNA levels and ~ 100 -fold reduction in tobacco NPTII levels. Therefore, it appears that accelerated mRNA turnover alone is insufficient to account for the reduction in NPTII accumulation. Reduced NPTII accumulation may in part be due to differential rates of *neo* mRNA

translation, as RNA sequences downstream of the AUG were shown to affect translation efficiency in chloroplasts (11). Since the *NheI* site in Nt-pHK40 plants is directly downstream of the AUG, a region important for translation efficiency, it is possible that the choice of restriction site at this position is important for high-level protein expression. Furthermore, the chimeric *neo* gene products differ with respect to the amino acid sequence of their N-terminal regions (Fig. 3). Thus, it is also possible that differential NPTII levels are due in part to differential rates of NPTII turnover.

The promoter of the rRNA operon is one of the strongest plastid promoters (9,35). Products of the operon are non-translated RNA species. A general approach to obtain protein expression from the *rrn* operon promoter has been fusion with translation control signals of plastid genes (17,36,37). A more complex approach involves engineering the entire translation control region including the 5'-UTR and DS sequences (11). The 23% NPTII reported here is the highest protein level obtained from a chloroplast transgene transcribed by a plastid RNA polymerase. Expression of β -glucuronidase at about the same level (20–30%) was reported from plastid genes transcribed by a nuclear-encoded, plastid-targeted phage T7 RNA polymerase. However, expression of the phage polymerase was detrimental to the plants (38).

There is significant interest in applying plant genetic engineering to the production of pharmaceutical compounds, vaccines and industrial enzymes (39–41). Thus far there are only a few examples of expression of economically important genes in chloroplasts (42–44). Transgenes utilizing the heterologous translation control signals described here are expected to perform better under field conditions than those utilizing native plastid signals as they are less likely to compete with native mRNAs for translation.

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REFERENCES

1. Sprengart, M.L., Fatscher, H.P. and Fuchs, E. (1990) The initiation of translation in *E. coli*: apparent base pairing between the 16s rRNA and downstream sequences of the mRNA. *Nucleic Acids Res.*, **18**, 1719–1723.
2. Sprengart, M.L., Fuchs, E. and Porter, A.G. (1996) The downstream box: an efficient and independent translation initiation signal in *Escherichia coli*. *EMBO J.*, **15**, 665–674.
3. Faxén, M., Plumbridge, J. and Isaksson, L.A. (1991) Codon choice and potential complementarity between mRNA downstream of the initiation codon and bases 1471–1480 in 16S ribosomal RNA affects expression of *glnS*. *Nucleic Acids Res.*, **19**, 5247–5251.
4. Etchegaray, J.P. and Inouye, M. (1999) Translational enhancement by an element downstream of the initiation codon in *Escherichia coli*. *J. Biol. Chem.*, **274**, 10079–10085.
5. Hirose, T., Kusumegi, T. and Sugiura, M. (1998) Translation of tobacco chloroplast *rps14* mRNA depends on a Shine–Dalgarno-like sequence in the 5'-untranslated region but not on internal RNA editing in the coding region. *FEBS Lett.*, **430**, 257–260.
6. Hirose, T. and Sugiura, M. (1996) Cis-acting elements and trans-acting factors for accurate translation of chloroplast *psbA* mRNAs: development of an *in vitro* translation system from tobacco chloroplasts. *EMBO J.*, **15**, 1687–1695.
7. Staub, J.M. and Maliga, P. (1993) Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the *psbA* mRNA. *EMBO J.*, **12**, 601–606.
8. Staub, J.M. and Maliga, P. (1994) Translation of *psbA* mRNA is regulated by light via the 5'-untranslated region in tobacco plastids. *Plant J.*, **6**, 547–553.
9. Shiina, T., Allison, L. and Maliga, P. (1998) *rbcL* transcript levels in tobacco plastids are independent of light: reduced dark transcription rate is compensated by increased mRNA stability. *Plant Cell*, **10**, 1713–1722.
10. Bonham-Smith, P.C. and Bourque, D.P. (1989) Translation of chloroplast-encoded mRNA: potential initiation and termination signals. *Nucleic Acids Res.*, **17**, 2057–2080.
11. Kuroda, H. and Maliga, P. (2001) Sequences downstream of the translation initiation codon are important determinants of translation efficiency in chloroplasts. *Plant Physiol.*, **125**, 430–436.
12. Stern, D.B., Higgs, D.C. and Yang, J.J. (1997) Transcription and translation in chloroplasts. *Trends Plant Sci.*, **2**, 308–315.
13. Bruick, R.K. and Mayfield, S.P. (1999) Light-activated translation of chloroplast mRNAs. *Trends Plant Sci.*, **4**, 190–195.
14. Nickelsen, J., Fleischmann, M., Boudreau, E., Rahire, M. and Rochaix, J.D. (1999) Identification of *cis*-acting RNA leader elements required for chloroplast *psbD* gene expression in *Chlamydomonas*. *Plant Cell*, **11**, 957–970.
15. Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.*, **185**, 60–89.
16. Vera, A. and Sugiura, M. (1995) Chloroplast rRNA transcription from structurally different tandem promoters: an additional novel-type promoter. *Curr. Genet.*, **27**, 280–284.
17. Svab, Z. and Maliga, P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl Acad. Sci. USA*, **90**, 913–917.
18. Chaudhuri, S. and Maliga, P. (1996) Sequences directing C to U editing of the plastid *psbL* mRNA are located within a 22 nucleotide segment spanning the editing site. *EMBO J.*, **15**, 5958–5964.
19. Staub, J.M. and Maliga, P. (1994) Extrachromosomal elements in tobacco plastids. *Proc. Natl Acad. Sci. USA*, **91**, 7468–7472.
20. Zoubenko, O.V., Allison, L.A., Svab, Z. and Maliga, P. (1994) Efficient targeting of foreign genes into the tobacco plastid genome. *Nucleic Acids Res.*, **22**, 3819–3824.
21. Murashige, T. and Skoog, F. (1962) A revised medium for the growth and bioassay with tobacco tissue culture. *Physiol. Plant.*, **15**, 473–497.
22. Stiekema, W.J., Heidekamp, F., Dirkse, W.G., van Beckum, J., de Haan, P., ten Bosch, C. and Louwerse, J.D. (1988) Molecular cloning and analysis of four potato tuber mRNAs. *Plant Mol. Biol.*, **11**, 255–269.
23. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
24. Svab, Z. and Maliga, P. (1991) Mutation proximal to the rRNA binding region of the *Nicotiana* plastid 16S rRNA confers resistance to spectinomycin. *Mol. Gen. Genet.*, **228**, 316–319.
25. Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.*, **60**, 512–538.
26. Kane, J.F. (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.*, **6**, 494–500.
27. Nakamura, Y., Gojobori, T. and Ikemura, T. (1999) Codon usage tabulated from the international DNA sequence databases; its status 1999. *Nucleic Acids Res.*, **27**, 292.
28. O'Connor, M., Asai, T., Squires, C.L. and Dahlberg, A.E. (1999) Enhancement of translation by the downstream box does not involve base pairing of mRNA with the penultimate stem sequence of the 16S rRNA. *Proc. Natl Acad. Sci. USA*, **96**, 8973–8978.
29. Kudla, J., Hayes, R. and Gruissem, W. (1996) Polyadenylation accelerates degradation of chloroplast mRNA. *EMBO J.*, **15**, 7137–7146.
30. Lisitsky, I., Klaff, P. and Schuster, G. (1996) Addition of destabilizing poly(A)-rich sequences to endonuclease cleavage sites during the degradation of chloroplast mRNA. *Proc. Natl Acad. Sci. USA*, **93**, 13398–13403.
31. Drager, R.G., Girard-Bascou, J., Choquet, Y., Kindle, K.L. and Stern, D.B. (1998) *In vivo* evidence for 5'→3' exoribonuclease degradation of an unstable chloroplast mRNA. *Plant J.*, **13**, 85–96.
32. Drager, R.G., Higgs, D.C., Kindle, K.L. and Stern, D.B. (1999) 5' to 3' exoribonucleolytic activity is a normal component of chloroplast mRNA decay pathways. *Plant J.*, **19**, 521–531.
33. Boudreau, E., Nickelsen, J., Lemaire, S.D., Ossendahl, F. and Rochaix, J.D. (2000) The *Nac2* gene of *Chlamydomonas* encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. *EMBO J.*, **19**, 3366–3376.

34. Higgs, D.C., Shapiro, R.S., Kindle, K.L. and Stern, D.B. (1999) Small *cis*-acting sequences that specify secondary structures in a chloroplast mRNA are essential for RNA stability and translation. *Mol. Cell. Biol.*, **19**, 8479–8491.
35. Baumgartner, B.J., Rapp, J.C. and Mullet, J.E. (1993) Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (*Hordeum vulgare*) chloroplast development: evidence for selective stabilization of *psbA* mRNA. *Plant Physiol.*, **101**, 781–791.
36. Carrer, H., Hockenberry, T.N., Svab, Z. and Maliga, P. (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Mol. Gen. Genet.*, **241**, 49–56.
37. Eibl, C., Zou, Z., Beck, A., Kim, M., Mullet, J. and Koop, H.U. (1999) *In vivo* analysis of plastid *psbA*, *rbcL* and *rpl32* UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. *Plant J.*, **19**, 333–345.
38. McBride, K.E., Schaaf, D.J., Daley, M. and Stalker, D.M. (1994) Controlled expression of plastid transgenes in plants based on a nuclear DNA-encoded and plastid-targeted T7 RNA polymerase. *Proc. Natl Acad. Sci. USA*, **91**, 7301–7305.
39. Ma, J.K. and Vine, N.D. (1999) Plant expression systems for the production of vaccines. *Curr. Top. Microbiol. Immunol.*, **236**, 275–292.
40. Hood, E.E. and Jilka, J.M. (1999) Plant-based production of xenogenic proteins. *Curr. Opin. Biotechnol.*, **10**, 382–386.
41. Arntzen, C.J. (1998) Pharmaceutical foodstuffs—oral immunization with transgenic plants. *Nature Med.*, **4** (suppl. 5), 502–503.
42. McBride, K.E., Svab, Z., Schaaf, D.J., Hogan, P.S., Stalker, D.M. and Maliga, P. (1995) Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Biotechnology*, **13**, 362–365.
43. Daniell, H., Datta, R., Varma, S., Gray, S. and Lee, S.B. (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nature Biotechnol.*, **16**, 345–348.
44. Staub, J.M., Garcia, B., Graves, J., Hajdukiewicz, P.T.J., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J.A., Ward, D., Ye, G. and Russel, D.A. (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature Biotechnol.*, **18**, 333–338.
45. Dams, E., Hendriks, L., Van de Peer, Y., Neefs, J.M., Smits, G., Vandembemt, I. and De Wachter, R. (1988) Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res.*, **16** (suppl.), r87–r173.
46. Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsabayashi, T., Zaita, N., Chungwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Deno, H., Kamogashira, T., Yamada, K., Kasuda, J., Takaiwa, F., Kato, A., Todoh, N., Shimada, H. and Sugiura, M. (1986) The complete sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J.*, **5**, 2043–2049.