Published online 16 August 2011 *Nucleic Acids Research, 2011, Vol. 39, No. 21* ***9405–9412***
doi:10.1093/nar/gkr644

The downstream *atpE* cistron is efficiently translated via its own *cis*-element in partially overlapping *atpB–atpE* dicistronic mRNAs in chloroplasts

Haruka Suzuki[1], Hiroshi Kuroda[1], Yasushi Yukawa[1] and Masahiro Sugiura[1,2,*]

[1]Graduate School of Natural Sciences, Nagoya City University, Yamanohata, Mizuho, Nagoya 467-8501 and [2]Sugiyama Human Research Center, Sugiyama Jogakuen University, Nagoya 464-8662, Japan

Received June 20, 2011; Revised and Accepted July 22, 2011

ABSTRACT

The chloroplast *atpB* and *atpE* genes encode subunits β and ε of the ATP synthase, respectively. They are co-transcribed as dicistronic mRNAs in flowering plants. An unusual feature is an overlap (AUGA) of the *atpB* stop codon (UGA) with the *atpE* start codon (AUG). Hence, *atpE* translation has been believed to depend on *atpB* translation (i.e. translational coupling). Using an *in vitro* translation system from tobacco chloroplasts, we showed that both *atpB* and *atpE* cistrons are translated from the tobacco dicistronic mRNA, and that the efficiency of *atpB* translation is higher than that of *atpE* translation. When the *atpB* 5′-UTR was replaced with lower efficiency 5′-UTRs, *atpE* translation was higher than *atpB* translation. Removal of the entire *atpB* 5′-UTR arrested *atpB* translation but *atpE* translation still proceeded. Introduction of a premature stop codon in the *atpB* cistron did not abolish *atpE* translation. These results indicate that *atpE* translation is independent of *atpB* translation. Mutation analysis showed that the *atpE* cistron possesses its own *cis*-element(s) for translation, located ∼25 nt upstream from the start codon.

INTRODUCTION

Chloroplasts carry their own genomes and gene expression systems. The chloroplast genome in flowering plants encodes about 80 proteins (1,2). Chloroplast gene expression is mainly regulated at post-transcriptional steps such as translation (3–10). Many protein-coding genes in flowering plant chloroplasts are clustered (11,12). These genes are co-transcribed to produce polycistronic transcripts that are then processed into mature mRNAs (1,13–17).

In the tobacco chloroplast genome, the protein-coding regions (hereafter cistrons) of eight genes partially overlap; these are *psbD–psbC*, *ndhC–ndhK*, *atpB–atpE* and *rpl22–rps3* (18). This feature implies that translation of a downstream gene depends on that of its upstream gene, namely translational coupling or termination-dependent translation, as reported for some genes from *Escherichia coli*, its bacteriophages and some eukaryotic viruses [reviewed in (19)]. A typical case of translational coupling in partially overlapping cistrons is explained by the downstream cistron being translated exclusively by the ribosomes that completed translation of the upstream cistron. In this case, a small fraction of the ribosomes participates in translating the downstream cistron and the majority of the ribosomes are released at the stop codon of the upstream cistron (20,21). Hence, translation of the downstream cistron is usually very low when compared to that of the upstream cistron. When the distance between the stop codon of an upstream cistron and the start codon of the downstream cistron increases, for example, by insertion of a premature termination codon in the upstream cistron, it abolishes translation of the downstream cistron.

The chloroplast ATP synthase consists of nine distinct subunits that are encoded in both the nuclear and chloroplast genomes: three in the nucleus (*atpC*, *atpD* and *atpG*) and six in the chloroplast (*atpA*, *atpB*, *atpE*, *atpF*, *atpH* and *atpI*) (22,23). The six subunits synthesized within chloroplasts are encoded by two separate operons: the *atpB–atpE* (*atpB/E*) operon and the *atpI–atpH–atpF–atpA* cluster. The *rps2* gene precedes the latter cluster and these five genes constitute an operon (24–27). In most flowering plants, the *atpB* stop codon (UGA) overlaps the *atpE* start codon (AUG), with the AUGA containing overlapping stop and start codons, and hence translation of these genes has been assumed to be coupled (28,29) (Figure 1). Expression of the maize chloroplast *atpB/E* genes in transformed *E. coli* and the cyanobacterium

*To whom correspondence should be addressed. Tel/Fax: +81 52 872 6021; Email: sugiura@nsc.nagoya-cu.ac.jp

Synechocystis sp. PCC6803 was examined, and translation of the downstream *atpE* cistron was reported to depend on translation of the upstream *atpB* cistron in these heterologous systems (30). In tobacco, the *atpE* gene is transcribed from its own promoter located within the *atpB* cistron to provide monocistronic mRNA (31) (Figure 1). This monocistronic *atpE* mRNA is translatable *in vitro* (32,33). However, translation of the *atpE* cistron from the dicistronic *atpB*/*E* mRNA has not been investigated.

Here, we show that the *atpE* cistron can also be translated from a dicistronic transcript using a chloroplast *in vitro* translation system. Unexpectedly, the majority of the translation of the *atpE* cistron does not depend on the upstream cistron; the *atpE* cistron is translated via its own *cis*-element(s). An upstream sequence of ~25 nt is necessary for efficient *atpE* translation.

MATERIALS AND METHODS

Chloroplast extracts

Growth of *Nicotiana tabacum* (var. Bright Yellow 4), isolation of intact chloroplasts and preparation of chloroplast extracts (S30 fractions) for *in vitro* translation were as described (34). An RNase inhibitor (Takara Bio, Otsu, Japan) was added during preparation of S30 fractions.

Plasmid construction and RNA synthesis

The mGFP-coding region in plasmid pHK309 (34), derived from pKH6 (35), was modified to create coding regions for Citrine (36) and enhanced GFP (EGFP) (37). Necessary parts of the *atpB*/*E*, *atpH* and *rbcL* sequences were amplified by PCR from tobacco chloroplast DNA (38). Strategies to construct plasmid DNAs are shown in Supplementary Figure S1. Deletion and point mutations were performed as described (39). A frameshift was introduced in the *atpB* cistron by inserting the PCR product of a frameshifted *atpB* segment into the SpeI/BglII region (Supplementary Figure S2). The resulting DNA fragments were confirmed to be the ones wanted by DNA sequencing. Plasmid DNAs were linearized with XhoI at the end of the 3′-UTRs and purified with a Wizard SV DNA Purification Kit (Promega, Madison, WI, USA). Test mRNAs were synthesized from the linearized DNAs using an AmpliScribe T7 Flash Transcription kit (Epicentre, Madison, WI, USA). After purification using the SV Total RNA isolation kit (Promega), the mRNAs were precipitated with ethanol and finally dissolved in nuclease-free water.

In vitro translation assays

Assays were carried out basically as described (34). Briefly, the 20-μl reaction mixture contained 30 mM HEPES/KOH (pH 7.7), 50 mM potassium acetate, 9 mM magnesium acetate, 60 mM ammonium acetate, 5 mM dithiothreitol, 40 μM of each of the 20 common amino acids, 2 mM ATP (lithium salt), 0.2 mM GTP (lithium salt), 8 mM phosphocreatine, 8 mg/ml creatine kinase, 12 A_{280} U/ml S30 fraction and 4 pmol test mRNA. After incubation for 1 h at 28°C, the reaction mixture was mixed with 4 μl of sample buffer (375 mM Tris–HCl, pH 6.8, 6% Triton X-100, 600 mM dithiothreitol, 30% glycerol, 50 μg/ml chloramphenicol). Protein products were separated by 12.5% native polyacrylamide gel electrophoresis (PAGE). Fluorescent signals were detected and quantified with a Typhoon 9400 Imager (GE Healthcare, Little Chalfont, UK) using 520BP40 and 555BP20 filters when excited by 488 and 532-nm actinic light, respectively.

RESULTS

Translation of the *atpE* cistron from dicistronic *atpB*/*E* mRNA

The tobacco chloroplast *atpE* gene is mainly co-transcribed with its upstream *atpB* gene as multiple *atpB*/*E* transcripts varying in size from 2.3 to 2.7 kb (31,40). Although the dicistronic transcript is expected to

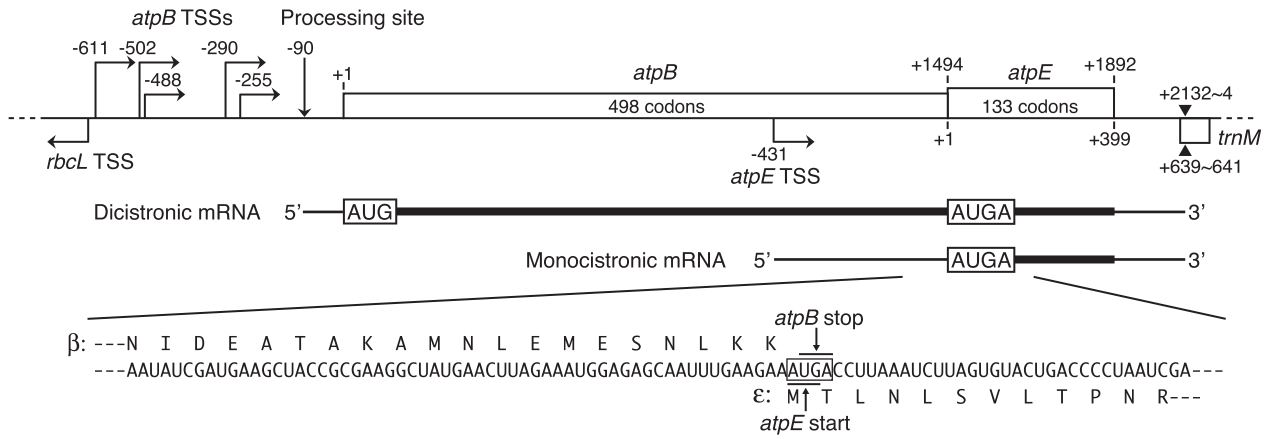

Figure 1. The tobacco chloroplast *atpB*/*atpE* operon and its transcripts. Nucleotide positions relative to AUG start codons (A as +1) are shown above for *atpB* and below for *atpE*. Bent arrows indicate *atpB* and *atpE* transcription start sites (TSSs) (31,42–44). A vertical arrow indicates a processing site (−90) (42). The common 3′-end is designated by closed triangles (31). AUG, *atpB* start codon; AUGA, overlapping *atpB* stop-*atpE* start codons. A partial mRNA sequence around the *atpE* start codon is shown below. Deduced amino acid sequences from *atpB* and *atpE* genes (β- and ε-subunits) are shown above and below the mRNA sequence, respectively.

be translated into the ε subunit (the *atpE* product), no direct evidence for this has been reported. The overlapping stop and start codons (AUGA) might hamper translation initiation of the downstream *atpE* cistron in tobacco chloroplasts.

To examine whether the *atpE* cistron is translated from the dicistronic mRNA, we designed test mRNAs from which translation of only the *atpE* cistron can be monitored. A large portion of the *atpE* cistron (133 codons) was replaced with the coding region (*citrine*) for Citrine (an improved version of yellow fluorescent protein) (36). Specifically, a *citrine*-coding sequence (237 codons without start codon) was fused 36 nt downstream from the *atpE* start codon, and then the tobacco chloroplast *rps16* 3′-UTR (150 nt) was added to stabilize the mRNA (41) (Figure 2A). The tobacco *atpB* gene has multiple transcription initiation sites (TSSs) and one processing site at position −90 (11,40,42–44) (Figure 1). We used the processed 5′-UTR of 90 nt because it was much more efficiently translated *in vitro* than the primary 5′-UTR (34). Monocistronic *atpE* mRNA was also prepared as a control. We left the first 36 nt of the *atpE* cistron in the construct because sequences downstream of start codons are sometimes necessary for efficient translation (45).

In vitro translation reaction was performed under template limiting (4 pmol/20 μl) and linear progression (1 h at 28°C) conditions, which enable estimation of the relative rate of translation (34). Test mRNAs in the reaction were stable for at least 2 h, probably due to the presence of an RNase inhibitor (34). After reaction, translation products were separated by native PAGE and the fluorescence intensity of Citrine products was detected by irradiation with 488 or 532-nm actinic light. As shown in Figure 2B, 488 and 532-nm excitation gave single fluorescent bands from both the dicistronic mRNA and the control monocistronic mRNA. This result clearly indicates that *atpE* translation occurs from the dicistronic mRNA.

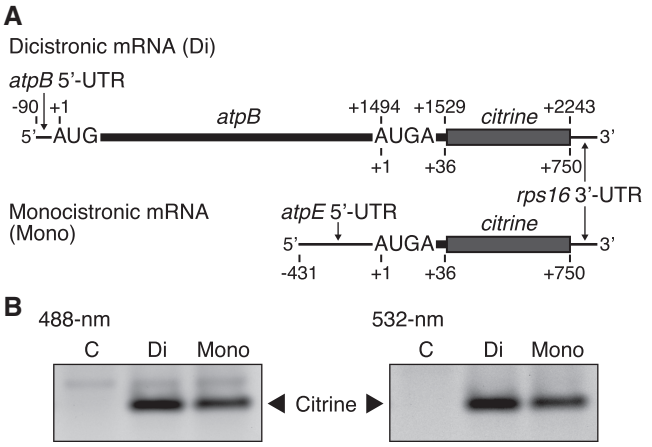

Figure 2. Translation of the *atpE* cistron from dicistronic and monocistronic mRNAs. (**A**) mRNA templates. A large portion of the *atpE* cistron was replaced by the Citrine-coding region (*citrine*). Positions are as in Figure 1. The full sequence is in Supplementary Figure S3. (**B**) Gel patterns of translation products. Synthesized Citrine products were detected by 488-nm light (left panel) and 532-nm light (right panel). Lanes labeled C, no-mRNA template control. Di and Mono indicate dicistronic and monocistronic mRNAs, respectively. Citrine bands represent *atpE* products (ε-subunit). Faint bands above the main Citrine bands are probably non-specific fluorescent substances in the S30 fraction.

Test for translational coupling of *atpB* and *atpE* cistrons

To examine whether translational coupling occurs, we devised an assay system to differentially monitor translation of the *atpB* and *atpE* cistrons. A large portion (from +4 to +1413, 470 codons) of the *atpB* cistron (498 codons) was replaced with a reading frame (236 codons without start and stop codons, *egfp*) for EGFP (37) in the dicistronic mRNA described above (Figure 3A). We left the last 81 nt of the *atpB* cistron in the construct because sequence upstream of the *atpE* start codon could be required for *atpE* translation initiation. Actinic light at 488 nm excites EGFP and Citrine, while 532-nm light excites only Citrine. After *in vitro* translation, two fluorescent bands were detected under 488-nm light while only the upper band was detected by 532-nm light (Figure 3B, lanes labeled *atpB*), indicating that the upper band corresponds to a Citrine product (from the *atpE* start codon) and the lower band to an EGFP product (from the *atpB* start codon). Hence, translation from *atpB* and *atpE* start codons can be differentially detected in the same gel. Based on the normalized intensity of EGFP and Citrine fluorescence, the efficiency of *atpE* translation was estimated to be ∼80% of *atpB* translation (Supplementary Figure S4).

If translational coupling occurs, the translation efficiency of the *atpE* cistron should depend on translation of the upstream *atpB* cistron. We replaced the *atpB* 5′-UTR with either the *atpH* 5′-UTR or the *rbcL* 5′-UTR, both of which have lower translation efficiencies than the *atpB* 5′-UTR (34). As expected, replacing the 5′-UTR resulted in significant reduction of *atpB* translation (EGFP bands) (Figure 3B, left panel). However, *atpE* translation (Citrine bands) decreased only slightly (Figure 3B, right panel). As a result, translation of the downstream *atpE* cistron became higher than that of the upstream *atpB* cistron. Furthermore, removal of the entire *atpB* 5′-UTR and the following AUG completely abolished *atpB* translation while no significant effect was observed on translation of the *atpE* cistron (Figure 3B, lanes '–'). If translational coupling occurs, *atpE* translation should be absent or much less than *atpB* translation. Hence, the translational coupling model cannot explain our present observations.

To confirm this result, we introduced a U to A point mutation at position −69 relative to the A (+1) of the *atpE* start codon, which created a premature stop codon, UAA, from UAU (Tyr), right after *egfp* (within the 3′-*atpB* cistron) (Figure 4A). In the mutant mRNA, the upstream *atpB* and the downstream *atpE* cistrons no longer overlap and are separated by a 69-nt spacer, which is enough to interfere with translational coupling (19). As shown in Figure 4B, no significant reduction in *atpE* translation was observed. Using the monocistronic mRNA as a control, the same point mutation hardly affected *atpE* translation

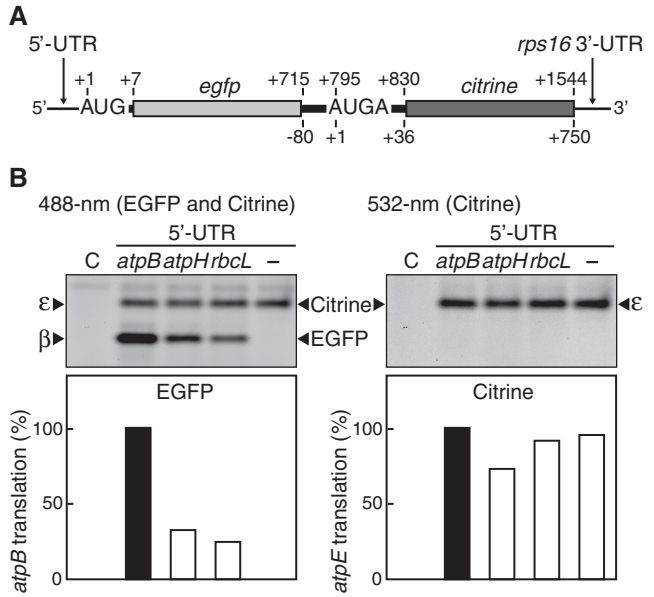

Figure 3. Effect of *atpB* translation on *atpE* translation. (**A**) mRNA templates with *atpB* and *atpE* cistrons replaced by EGFP (*egfp*) and Citrine (*citrine*)-coding regions, respectively. Positions are as in Figure 1. The full sequence is in Supplementary Figure S5. (**B**) Gel patterns of translation products. Lanes labeled C, no-mRNA control. The 5′-UTR was either from *atpB*, *atpH* or *rbcL*, and lane '–' denotes no 5′-UTR or following AUG (Supplementary Figure S5). Synthesized EGFP and Citrine products were detected by 488-nm light (left panel) and 532-nm light (right panel). EGFP and Citrine bands represent *atpB* and *atpE* products (β- and ε-subunits), respectively. Quantification of translation products based on intensity of fluorescent bands (*atpB* value defined as 100%) is shown in bar graphs below. Faint bands above the main EGFP bands could be loosely folded EGFP.

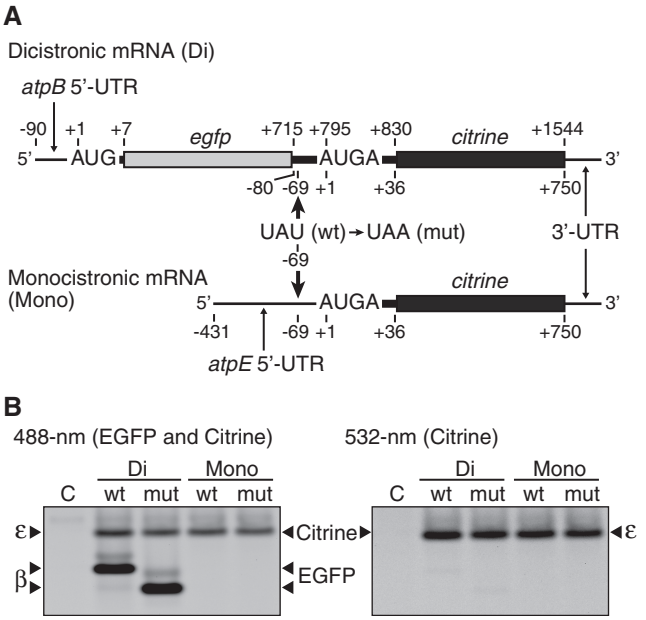

Figure 4. Effect of premature termination of the upstream *atpB* cistron on *atpE* translation. (**A**) mRNA templates as in Figures 3A (Di) and 2A (Mono). Bold arrows indicate locations of premature termination codons. The last U (position −69) of UAU was replaced with A to create a stop codon (UAA) in the 3′ *atpB* cistron or in the *atp*E 5′-UTR. (**B**) Gel patterns of translation products. Lanes labeled C, no-mRNA control. Di and Mono indicate dicistronic and monocistronic mRNAs, respectively. 'wt', wild-type (UAU); 'mut', mutant (UAG). Synthesized EGFP and Citrine products were detected as in Figure 3. The EGFP product from the wt mRNA includes a β subunit fragment (27 amino acids) at the C-terminus. The premature termination deleted 24 amino acids from this extension, and therefore the band migrated faster. Faint bands above the main EGFP bands could be loosely folded EGFP.

(Figure 4B, lanes labeled 'Mono'). Similarly, *atpB* translation was not influenced by premature termination, though the size of EGFP products was reduced (Figure 4B, left panel).

To exclude the possibility that *egfp* interferes with translational coupling, we introduced a frameshift mutation in the middle of the original *atpB* cistron (insertion of UCGA between the 134th and the 135th codons at a BglII site), creating a premature stop codon (UGA) at the 151st codon (Figure 5A). Again, no significant inhibition of *atpE* translation was observed (Figure 5B). This mutation is identical to that applied to the maize chloroplast *atpB*/*E* operon expressed in *E. coli* cells, which caused almost complete prevention of expression of the *atpE* cistron (30). Taking this information together, we concluded that the downstream *atpE* cistron is translated almost independently from translation of the upstream *atpB* cistron (with little or no translational coupling) in the homologous chloroplast system. Therefore, the *atpE* cistron should be translated via its own *cis*-element.

Cis-elements required for *atpE* translation

To define the sequences required for translation of the *atpE* cistron from dicistronic mRNAs, we prepared a series of dicistronic mRNAs with internal deletions and assayed their translation. Step-wise deletions (3 nt each) in the 5′ *atpE* cistron (from +6 to +36) hardly affected translation of either the *atpB* or *atpE* cistron (Supplementary Figure S6), indicating that the *cis*-element is localized upstream of the *atpE* start codon. Figure 6A shows parts deleted in the region upstream (from −80 to −3) of the *atp*E start codon (hereafter *atp*E 5′-UTR). As shown in Figure 6B (lower panel), no significant reduction of *atpE* translation was observed by deletion up to −20, while *atpE* translation dropped with a further 3-nt deletion (−17 mRNA), by which the SD-like sequence (from −18 to −15) was truncated (GGAG to cGAG, c from the *egfp* 3′-end) (Figure 6A). Further deletions to −11 led to no additional decrease in *atpE* translation, but deletions from −8 to −2 significantly inhibited its translation, though not completely. These observations indicate that the sequence, at least from −18 to −2, is important for *atpE* translation. Translation of the *atpB* cistron was hardly affected by these deletions (Figure 6B, bold bands in upper panel). Note that migration of EGFP products in native gels depends largely on their sizes and charges. 5′-Deletion analysis of the monocistronic *atpE* mRNA showed that a region of ∼30 nt beginning 1 nt upstream from the start codon (−1) is also important for *atpE* translation (Supplementary Figure S7).

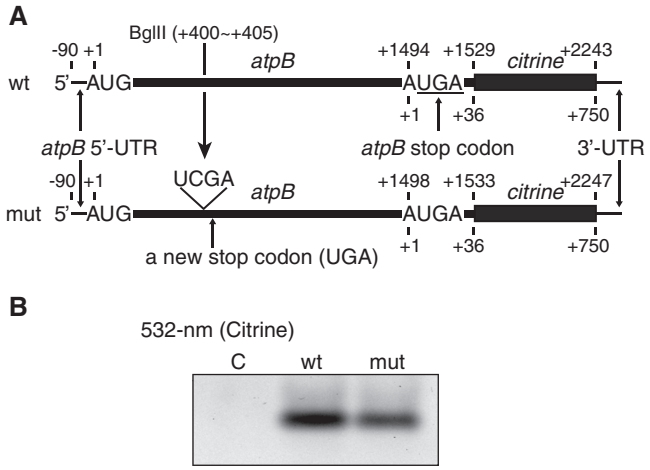

Figure 5. Effect of a frameshift mutation in the *atpB* cistron on *atpE* translation. (**A**) mRNA templates (as in Figure 2A) and location of the frameshift. Insertion of 4 nt at the first BglII site caused a frameshift, creating a stop codon within the *atpB* cistron. 'wt', wild type; 'mut', frameshift mutant. Sequences around the frameshift are in Supplementary Figure S2. (**B**) Gel patterns of translation products. Lanes labeled C, no-mRNA control. Synthesized Citrine products were detected by 532-nm light.

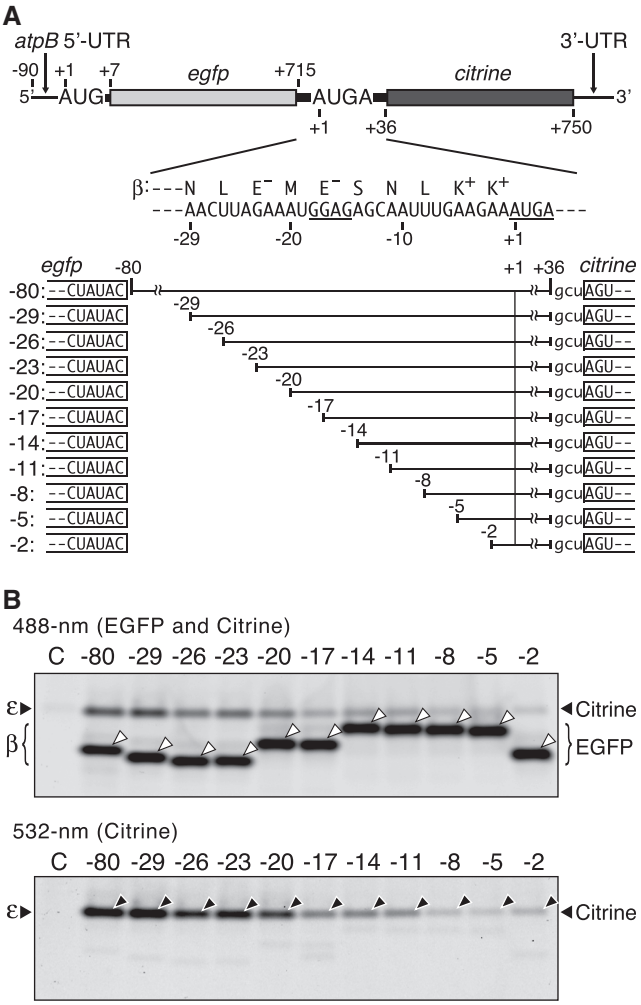

Figure 6. Effect of internal deletions in the *atpE* 5′-UTR on *atpE* translation. (**A**) The mRNA (as in Figure 3A) and partial *atpE* 5′-UTR sequence. The SD-like sequence and the overlapping stop and start codons are underlined. mRNAs with internal deletions (indicated as blanks, from −80 to −3) are shown below. Terminal sequences of *egfp* and *citrine* are boxed. The gcu triplet after +36 is a ligation product. (**B**) Gel patterns of translation products. Synthesized EGFP and Citrine products were detected by 488-nm light (upper panel) and 532-nm light (lower panel). White arrowheads in the upper panel point to EGFP bands and black arrowheads in the lower panel point to Citrine bands. In the upper panel, migration of EGFP bands differed due to charge and size differences on native gels; i.e. deletion of one negatively charged glutamate (E^-) caused slower migration ('−23' to '-20' and '−17' to '−14') and deletion of positively charged lysine (K^+) caused faster migration ('−5' to '−2').

We then introduced mutations in the *atpE* 5′-UTR (positions −25 to −5) of both dicistronic and monocistronic mRNAs (Figure 7A) and assayed translation. Mutation from UAG to CGA (m1, −25 to −23) partly inhibited *atpE* translation and mutation from AAAU to GGGC (m2, −22 to −19) severely impaired translation from both dicistronic and monocistronic mRNAs (Figure 7B, lower panel, lanes m1 and m2). As expected, mutation of the SD-like sequence GGAG to AAGA (m3, −18 to −15) strongly inhibited *atpE* translation from both mRNAs (lanes m3; ∼5-fold decrease in dicistronic and ∼10-fold decrease in monocistronic mRNAs). Changes from AA to GG (m5, −11 to −10), UU to CC (m6, −9 to −8) and UGA to CAG (m7, −7 to −5) also inhibited *atpE* translation (lower panel, lanes m5, m6 and m7). Inhibitory effects of these mutations were less in the dicistronic mRNA than in the monocistronic mRNA (lower panel). These mutations caused no significant effect on *atpB* translation (Figure 7B, left upper panel). Migration of EGFP products (bold bands) differed largely due to charge differences in native gels (see Supplementary Figure S8 for altered amino acid sequences). Together with the results shown in Figures 6 and 7, this shows that the upstream sequence at least 25 nt from the *atpE* start codon plays an important role in efficient translation initiation of the *atpE* cistron in both dicistronic and monocistronic mRNAs.

Internal deletion up to −20 caused no significant inhibition of *atpE* translation (Figure 6B, lower panel, lanes −29 to −20), whereas mutations between −25 and −20 caused inhibition (Figure 7, lanes m1 and m2). The internal deletion resulted in fusion of the 3′-end of *egfp* (UAUAC, −85 to −81 in Figure 6A) to position −20, which could mimic the original sequence (UAGAA, −25 to −21), as these two sequences are similar. Mutation from AGC to GAU (−14 to −12, lane m4) caused no apparent inhibition from either dicistronic or monocistronic mRNAs. These observations suggest that either some nucleotide changes within the 25 nt sequence are tolerable as a regulatory sequence for *atpE* translation initiation or there are two separate *cis*-elements.

Deletion from positions −80 to −3 in the *atp*E 5′-UTR did not completely inhibit *atpE* translation (Figure 6B, lanes −5 and −2). Furthermore, mutation m2 abolished *atpE* translation from monocistronic mRNA whereas slight *atp*E translation was observed from dicistronic

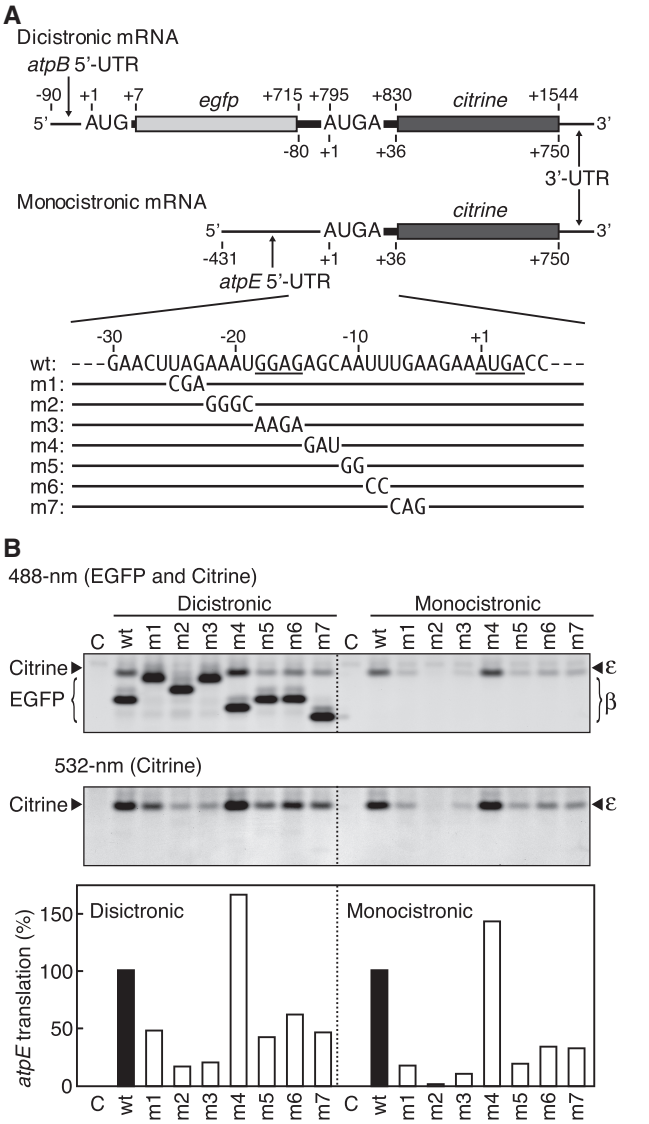

Figure 7. Effect of mutations in the *atpE* 5′-UTR on *atpE* translation. (**A**) mRNA templates as in Figures 3A (Dicistronic) and 2A (Monocistronic) and partial *atpE* 5′-UTR sequence. The SD-like sequence and the overlapping stop and start codons are underlined. Nucleotide and amino acid sequences are in Supplementary Figure S8. (**B**) Gel patterns of translation products. Lanes labeled C, no-mRNA control. Synthesized EGFP and Citrine products were detected as in Figure 6. In the upper left panel, migration of EGFP bands differed due to charge differences on native gels. The m1 and m3 dense bands overlap Citrine and EGFP products. Quantification of Citrine products based on intensity of fluorescent bands (wt value defined as 100%) is shown in bar graphs below.

mRNA (Figure 7B, lanes m2). The residual translation of the *atpE* cistron from the dicistronic mRNA may be dependent on the upstream *atpB* cistron (see 'Discussion' section).

DISCUSSION

The chloroplast genes for ATP synthase subunits β and ε in many flowering plants possess overlapping start and stop codons. Based on this structure, it has been assumed that translation of these genes is coupled. Gatenby *et al.* (30) reported that the maize chloroplast *atpB* and *atpE* genes are translationally coupled in transformed *E. coli* and *Synechocystis* cells. They used these heterologous systems because no *in vitro* translation system from chloroplasts existed. We have established a highly active *in vitro* translation system from tobacco chloroplasts (34). Using this system, we showed that translational coupling does occur in the partially overlapping tobacco *ndhC–ndhK* transcripts (46) and in the overlapping tobacco *psbD–psbC* mRNA (Y. Adachi, H. Kuroda, Y. Yukawa and M. Sugiura).

However, unexpectedly, we concluded that the tobacco *atpB/E* mRNAs are not translationally coupled (except perhaps for a minor fraction) in the homologous tobacco chloroplast translation system. To arrive at this conclusion, we first devised an assay to detect translation from two cistrons differentially using two different fluorescent proteins. Results obtained by exchanging 5′-UTRs (Figure 3) and introduction of premature stop codons (Figures 4 and 5) indicated that no translational coupling occurs between the two cistrons.

Translation of the tobacco *atpE* cistron should occur via its own *cis*-element. Deletion and mutation analyses showed that the *cis*-element(s) resides within 25 nt or so from the *atpE* start codon. We previously reported that the SD-like sequence is required for translation of the monocistronic *atpE* mRNA because changing the GGAG to CCUC reduced *atpE* translation to <5% of the original mRNA (33). This was confirmed by our present study (see Figure 6, lane −17 and Figure 7, lane m3). However, our present study showed that the SD-like sequence is not the sole determinant. A sequence of at least 25 nt (up to −29) encompassing the SD-like sequence is necessary for efficient *atpE* translation from both dicistronic and monocistronic mRNAs (Figures 6 and 7). Therefore, the GGAG may not function similarly to the SD sequence found in most *E. coli* mRNAs. Baecker *et al.* (47) analyzed translation of chimeric *uidA* with the tobacco chloroplast *atpI* 5′-UTR in transplastomic tobacco plants. They found that efficient translation of *uidA* requires element(s) upstream of the putative SD sequence. Our results were similar. Translation of the tobacco *atpE* cistron may also require two or more *cis*-elements. In spinach chloroplasts, the *atpB/E* transcript lacking its 5′-UTR was found in crude polysomal fractions (48). Our study has clearly indicated that removal of the *atpB* 5′-UTR completely arrests translation of the *atpB* cistron but not the *atpE* cistron (see Figure 3, lanes '–'). Hence, this leaderless transcript is likely to support only *atpE* translation.

Chloroplast translation is usually regulated by positive *trans*-acting regulatory proteins (8,10,49,50). The maize nuclear *atp1* gene product is required for translation of the maize *atpB/E* mRNA (51). The tobacco chloroplast *atpB* mRNA contains no SD-like sequence but does contain a U-rich sequence in the 5′-UTR (−25 to −1), and this 25 nt upstream sequence was essential for translation of the *atpB* cistron (32). A 50-kDa protein (p50), detected by UV-crosslinking, specifically bound the 25 nt sequence, suggesting that p50 is a *trans*-acting factor or a

component of such a factor for *atpB* translation initiation. One possibility is that p50 is the homolog of the maize *atp1* gene product. Specific chloroplast proteins were reported to bind to the *atpE* 5′-UTR as well as to the *atpB* 5′-UTR from spinach chloroplast mRNAs (52). Therefore, it is possible that a *trans*-acting factor(s) is also necessary for *atpE* translation. If this is the case, this factor binds to the *atpE* 5′-UTR, namely the 3′ side of the *atpB* coding region, which would hamper the elongation of *atpB* translation. It may be that a fraction of *atpB*/*E* mRNAs produces solely subunit β and the other fraction provides subunit ε.

Our study showed that the residual *atpE* translation from the dicistronic mRNA that may be due to translational coupling is <10% of *atpB* translation (Figures 6 and 7). The SD sequence is necessary to translate downstream cistrons by translational coupling in *E. coli* (19). However, residual translation was still observed after deletion and mutation of the SD-like sequence in the *atpE* 5′-UTR. Thus, we cannot rule out the possibility that an unknown mechanism supports this residual *atpE* translation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEGEMENTS

The authors thank their laboratory members for help and discussion.

FUNDING

Nagoya City University Funds; New Energy and Industrial Technology Development Organization; Grants-in-Aid for Scientific Research (15370025, 17370020 to M.S., 20570043 to H.K.). Funding for open access charge: Grant-in-Aid for Scientific Research (1737002).

Conflict of interest statement. None declared.

REFERENCES

1. Sugiura,M. (1992) The chloroplast genome. *Plant Mol. Biol.*, **19**, 149–168.
2. Bock,R. (2007) Structure, function, and inheritance of plastid genomes. In Bock,R. (ed.), *Cell and Molecular Biology of Plastids*, Vol. 19. Springer, Potsdam-Golm, pp. 29–63.
3. Deng,X.W. and Gruissem,W. (1987) Control of plastid gene expression during development: the limited role of transcriptional regulation. *Cell*, **49**, 379–387.
4. Mullet,J.E. (1988) Chloroplast development and gene expression. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **39**, 475–502.
5. Marín-Navarro,J., Manuell,A.L., Wu,J. and Mayfield,S.P. (2007) Chloroplast translation regulation. *Photosynth. Res.*, **94**, 359–374.
6. Wobbe,L., Schwarz,C., Nickelsen,J. and Kruse,O. (2008) Translational control of photosynthetic gene expression in phototrophic eukaryotes. *Physiol. Plant*, **133**, 507–515.
7. Sugiura,M., Hirose,T. and Sugita,M. (1998) Evolution and mechanism of translation in chloroplasts. *Annu. Rev. Genet.*, **32**, 437–459.
8. Peled-Zehavi,H. and Danon,A. (2007) Translation and translational regulation in chloroplasts. In Bock,R. (ed.), *Cell and Molecular Biology of Plastids*, Vol. 19. Springer, Berlin Heidelberg, pp. 248–281.
9. Gruissem,W. and Tonkyn,J.C. (1993) Control mechanisms of plastid gene expression. *Crit. Rev. Plant Sci.*, **12**, 19–55.
10. Rochaix,J.-D. (2006) The role of nucleus- and chloroplast-encoded factors in the synthesis of the photosynthetic apparatus. In Wise,R.R. and Hoober,J.K. (eds), *The Structure and Function of Plastids*, Vol. 23. Springer, Dordrecht, pp. 145–165.
11. Shinozaki,K. and Sugiura,M. (1982) Sequence of the intercistronic region between the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit and coupling factor β subunit gene. *Nucleic Acids Res.*, **10**, 4923–4934.
12. Hiratsuka,J., Shimada,H., Whittier,R., Ishibashi,T., Sakamoto,M., Mori,M., Kondo,C., Honji,Y., Sun,C.R., Meng,B.Y. *et al.* (1989) The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. *Mol. Gen. Genet.*, **217**, 185–194.
13. Tanaka,M., Obokata,J., Chunwongse,J., Shinozaki,K. and Sugiura,M. (1987) Rapid splicing and stepwise processing of a transcript from the *psbB* operon in tobacco chloroplasts: determination of the intron sites in *petB* and *petD*. *Mol. Gen. Genet.*, **209**, 427–431.
14. Matsubayashi,T., Wakasugi,T., Shinozaki,K., Yamaguchi-Shinozaki,K., Zaita,N., Hidaka,T., Meng,B.Y., Ohto,C., Tanaka,M., Kato,A. *et al.* (1987) Six chloroplast genes (*ndhA-F*) homologous to human mitochondrial genes encoding components of the respiratory chain NADH dehydrogenase are actively expressed: determination of the splice sites in *ndhA* and *ndhB* pre-mRNAs. *Mol. Gen. Genet.*, **210**, 385–393.
15. Barkan,A. (1988) Proteins encoded by a complex chloroplast transcription unit are each translated from both monocistronic and polycistronic mRNAs. *EMBO J.*, **7**, 2637–2644.
16. Westhoff,P. and Herrmann,R.G. (1988) Complex RNA maturation in chloroplasts. The *psbB* operon from spinach. *Eur. J. Biochem.*, **171**, 551–564.
17. Stern,D.B., Goldschmidt-Clermont,M. and Hanson,M.R. (2010) Chloroplast RNA metabolism. *Annu. Rev. Plant Biol.*, **61**, 125–155.
18. Shinozaki,K., Ohme,M., Tanaka,M., Wakasugi,T., Hayashida,N., Matsubayashi,T., Zaita,N., Chunwongse,J., Obokata,J., Yamaguchi-Shinozaki,K. *et al.* (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J.*, **5**, 2043–2049.
19. Jackson,R.J., Kaminski,A. and Pöyry,T.A.A. (2007) Coupled termination-reinitiation events in mRNA translation. In Mathews,M.B., Sonenberg,N. and Hershey,J.W.B. (eds), *Translational Control in Biology and Medicine*. Cold Spring Harbor Laboratory Press, New York, pp. 197–223.
20. Inokuchi,Y., Hirashima,A., Sekine,Y., Janosi,L. and Kaji,A. (2000) Role of ribosome recycling factor (RRF) in translational coupling. *EMBO J.*, **19**, 3788–3798.
21. Powell,M.L., Napthine,S., Jackson,R.J., Brierley,I. and Brown,T.D. (2008) Characterization of the termination-reinitiation strategy employed in the expression of influenza B virus BM2 protein. *RNA*, **14**, 2394–2406.
22. Hennig,J. and Herrmann,R.G. (1986) Chloroplast ATP synthase of spinach contains nine nonidentical subunit species, six of which are encoded by plastid chromosomes in two operons in a phylogenetically conserved arrangement. *Mol. Gen. Genet.*, **203**, 117–128.
23. Wollman,F.-A., Minai,L. and Nechushtai,R. (1999) The biogenesis and assembly of photosynthetic proteins in thylakoid membranes. *Biochim. Biophys. Acta*, **1411**, 21–85.
24. Hudson,G.S., Mason,J.G., Holton,T.A., Koller,B., Cox,G.B., Whitfeld,P.R. and Bottomley,W. (1987) A gene cluster in the spinach and pea chloroplast genomes encoding one CF_1 and three CF_0 subunits of the H^+-ATP synthase complex and the ribosomal protein S2. *J. Mol. Biol.*, **196**, 283–298.

25. Stollar,N.N., Kim,J.-K. and Hollingsworth,M.J. (1994) Ribosomes pause during the expression of the large ATP synthase gene cluster in spinach chloroplasts. *Plant Physiol.*, **105**, 1167–1177.
26. Miyagi,T., Kapoor,S., Sugita,M. and Sugiura,M. (1998) Transcript analysis of the tobacco plastid operon *rps2/atpI/H/F/A* reveals the existence of a non-consensus type II (NCII) promoter upstream of the *atpI* coding sequence. *Mol. Gen. Genet.*, **257**, 299–307.
27. Green,C.D. and Hollingsworth,M.J. (1992) Expression of the large ATP synthase gene cluster in spinach plastids during light-induced development. *Plant Physiol.*, **100**, 1164–1170.
28. Zurawski,G., Bottomley,W. and Whitfeld,P.R. (1982) Structures of the genes for the β and ε subunits of spinach chloroplast ATPase indicate a dicistronic mRNA and an overlapping translation stop/start signal. *Proc. Natl Acad. Sci. USA*, **79**, 6260–6264.
29. Krebbers,E.T., Larrinua,I.M., McIntosh,L. and Bogorad,L. (1982) The maize chloroplast genes for the β and ε subunits of the photosynthetic coupling factor CF_1 are fused. *Nucleic Acids Res.*, **10**, 4985–5002.
30. Gatenby,A.A., Rothstein,S.J. and Nomura,M. (1989) Translational coupling of the maize chloroplast *atpB* and *atpE* genes. *Proc. Natl Acad. Sci. USA*, **86**, 4066–4070.
31. Kapoor,S., Wakasugi,T., Deno,H. and Sugiura,M. (1994) An *atpE*-specific promoter within the coding region of the *atpB* gene in tobacco chloroplast DNA. *Curr. Genet.*, **26**, 263–268.
32. Hirose,T. and Sugiura,M. (2004) Multiple elements required for translation of plastid *atpB* mRNA lacking the Shine-Dalgarno sequence. *Nucleic Acids Res.*, **32**, 3503–3510.
33. Hirose,T. and Sugiura,M. (2004) Functional Shine-Dalgarno-like sequences for translational initiation of chloroplast mRNAs. *Plant Cell Physiol.*, **45**, 114–117.
34. Yukawa,M., Kuroda,H. and Sugiura,M. (2007) A new *in vitro* translation system for non-radioactive assay from tobacco chloroplasts: effect of pre-mRNA processing on translation *in vitro*. *Plant J.*, **49**, 367–376.
35. Hayashi,K., Shiina,T., Ishii,N., Iwai,K., Ishizaki,Y., Morikawa,K. and Toyoshima,Y. (2003) A role of the -35 element in the initiation of transcription at *psbA* promoter in tobacco plastids. *Plant Cell Physiol.*, **44**, 334–341.
36. Heikal,A.A., Hess,S.T., Baird,G.S., Tsien,R.Y. and Webb,W.W. (2000) Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: coral red (dsRed) and yellow (Citrine). *Proc. Natl Acad. Sci. USA*, **97**, 11996–12001.
37. Cormack,B.P., Valdivia,R.H. and Falkow,S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, **173**, 33–38.
38. Yukawa,M., Tsudzuki,T. and Sugiura,M. (2005) The 2005 version of the chloroplast DNA sequence from tobacco (*Nicotiana tabacum*). *Plant Mol. Biol. Rep.*, **23**, 1–7.
39. Kuroda,H., Suzuki,H., Kusumegi,T., Hirose,T., Yukawa,Y. and Sugiura,M. (2007) Translation of *psbC* mRNAs starts from the downstream GUG, not the upstream AUG, and requires the extended Shine-Dalgarno sequence in tobacco chloroplasts. *Plant Cell Physiol.*, **48**, 1374–1378.
40. Shinozaki,K., Deno,H., Kato,A. and Sugiura,M. (1983) Overlap and cotranscription of the genes for the beta and epsilon subunits of tobacco chloroplast ATPase. *Gene*, **24**, 147–155.
41. 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.
42. Orozco,E.M.J., Chen,L.J. and Eilers,R.J. (1990) The divergently transcribed *rbcL* and *atpB* genes of tobacco plastid DNA are separated by nineteen base pairs. *Curr. Genet.*, **17**, 65–71.
43. Kapoor,S., Suzuki,J.Y. and Sugiura,M. (1997) Identification and functional significance of a new class of non-consensus-type plastid promoters. *Plant J.*, **11**, 327–337.
44. Hajdukiewicz,P.T.J., Allison,L.A. and Maliga,P. (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *EMBO J.*, **16**, 4041–4048.
45. 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.
46. Yukawa,M. and Sugiura,M. (2008) Termination codon-dependent translation of partially overlapping *ndhC-ndhK* transcripts in chloroplasts. *Proc. Natl Acad. Sci. USA*, **105**, 19550–19554.
47. Baecker,J.J., Sneddon,J.C. and Hollingsworth,M.J. (2009) Efficient translation in chloroplasts requires element(s) upstream of the putative ribosome binding site from *atpI*. *Am. J. Bot.*, **96**, 627–636.
48. Bennett,D.C., Rogers,S.A., Chen,L.-J. and Orozco,E.M. Jr. (1990) A primary transcript in spinach chloroplasts that completely lacks a 5′ untranslated leader region. *Plant Mol. Biol.*, **15**, 111–119.
49. Goldschmidt-Clermont,M. (1998) Coordination of nuclear and chloroplast gene expression in plant cells. *Int. Rev. Cytol.*, **177**, 115–180.
50. Nickelsen,J. (2003) Chloroplast RNA-binding proteins. *Curr. Genet.*, **43**, 392–399.
51. McCormac,D.J. and Barkan,A. (1999) A nuclear gene in maize required for the translation of the chloroplast *atpB/E* mRNA. *Plant Cell*, **11**, 1709–1716.
52. Robida,M.D., Merhige,P.M. and Hollingsworth,M.J. (2002) Proteins are shared among RNA-protein complexes that form in the 5′ untranslated regions of spinach chloroplast mRNAs. *Curr. Genet.*, **41**, 53–62.