Both RNA editing and RNA cleavage are required for translation of tobacco chloroplast *ndhD* mRNA: a possible regulatory mechanism for the expression of a chloroplast operon consisting of functionally unrelated genes

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Tobacco chloroplast genes encoding a photosystem I component (*psaC*) and a NADH dehydrogenase subunit (*ndhD*) are transcribed as a dicistronic pre-mRNA which is then cleaved into short mRNAs. An RNA protection assay revealed that the cleavage occurs at multiple sites in the intercistronic region. There are two possible initiation codons in the tobacco *ndhD* mRNA: the upstream AUG and the AUG created by RNA editing from the in-frame ACG located 25 nt downstream. Using the chloroplast *in vitro* translation system, we found that translation begins only from the edited AUG. The extent of ACG to AUG editing is partial and depends on developmental and environmental conditions. In addition, the *in vitro* assay showed that the *psaC/ndhD* dicistronic mRNA is not functional and that the intercistronic cleavage is a prerequisite for both *ndhD* and *psaC* translation. Using a series of mutant mRNAs, we showed that an intramolecular interaction between an 8 nt sequence in the *psaC* coding region and its complementary 8 nt sequence in the 5' *ndhD* UTR is the negative element for translation of the dicistronic mRNA. A possible mechanism in which the differential expression of the chloroplast operon consists of functionally unrelated genes is discussed.

Keywords: chloroplast/RNA cleavage/RNA editing/RNA–RNA interaction/translation

Introduction

Chloroplasts have their own genome and expression system. Chloroplast gene expression is regulated at the level of transcription, RNA processing and translation during development, and in response to environmental cues (reviewed in Gruissem, 1989; Rochaix, 1992; Gruissem and Tonkyn, 1993; Mullet, 1993; Mayfield *et al.*, 1996; Sugita and Sugiura, 1996). In higher plant chloroplasts, many chloroplast genes are primarily transcribed as polycistronic forms, and these pre-mRNAs are endonucleolytically processed into complex sets of overlapping transcripts, including monocistronic mRNAs, during which some of the transcripts are edited and/or spliced (Herrmann *et al.*, 1985; Tanaka *et al.*, 1987; Barkan, 1988; Westhoff and Herrmann, 1988; Neuhaus and Link, 1990; Sexton *et al.*, 1990; Hird *et al.*, 1991; Hoch *et al.*, 1991). RNA processing has been recognized as one of the important regulatory steps in chloroplast gene expression (Deng and

Gruissem, 1988; Barkan, 1989; Herrmann *et al.*, 1992; Rochaix, 1992; Sugiura, 1992; Gruissem and Tonkyn, 1993; Mayfield *et al.*, 1996). Cleavage of some chloroplast pre-mRNAs is required to form functional mRNAs: for example, alternative cleavage of the 5' untranslated region induced by methyl-jasmonate-impaired translation of the *rbcL* transcript in barley (Reinbothe *et al.*, 1993), and one nuclear mutation in maize blocked both the cleavage and efficient translation of several chloroplast transcripts (Barkan *et al.*, 1994). On the other hand, it was reported that the GUS gene, which is integrated by plastid transformation in the chloroplast genome downstream of the *rbcL* gene, is transcribed together with *rbcL* and the transcript is mainly accumulated as dicistronic mRNAs from which GUS is efficiently translated in tobacco chloroplasts (Staub and Maliga, 1995). This result implies that intercistronic cleavage of pre-mRNAs is not always necessary for translation.

Instances of RNA editing so far found in chloroplasts of angiosperms and gymnosperms are rare, and all events are C-to-U substitutions which mostly restore internal codons for conserved amino acids (e.g. Maier *et al.*, 1992) or create initiation codons (e.g. Hoch *et al.*, 1991) or termination codons (Wakasugi *et al.*, 1996). Recently, however, extensive RNA editing, including U-to-C changes, has been reported in the *rbcL* transcripts from the chloroplasts of hornwort *Anthoceros formosae* (a bryophyte) (Yoshinaga *et al.*, 1996). The extent of editing in several chloroplast transcripts has been shown to vary according to plastid types or light conditions, suggesting that RNA editing is an additional regulatory step for chloroplast gene expression (Bock *et al.*, 1993; Hirose *et al.*, 1996).

In addition to multiple RNA processing steps, translation is also an important regulatory step for gene expression in higher plant chloroplasts. For example, expression of the *psbA* gene encoding the D1 protein of photosystem II has been shown to be regulated at the step of translational initiation and elongation during plastid development or by a light–dark transition (Deng and Gruissem, 1988; Klein *et al.*, 1988; Staub and Maliga, 1993; Taniguchi *et al.*, 1993; Kim and Mullet, 1994; Hirose *et al.*, 1995; Kuroda *et al.*, 1996). It was also reported that translational initiation of *rbcL* mRNAs is regulated by light or developmental conditions in *Amaranthus* and spinach (Berry *et al.*, 1988; Deng and Gruissem, 1988). In *Chlamydomonas*, nearly all chloroplast genes appear to be transcribed as monocistronic mRNAs, and translation is one of the key steps in the regulation of gene expression (e.g. Rochaix *et al.*, 1989; Danon and Mayfield, 1994; Sakamoto *et al.*, 1994; Zerger and Rochaix, 1994).

In order to elucidate the molecular mechanism of translational regulation in higher plant chloroplasts, we

have developed an *in vitro* translation system from tobacco chloroplasts, and shown the existence of novel *cis*-acting regulatory elements and *trans*-acting factor(s) for accurate translation of *psbA* mRNA (Hirose and Sugiura, 1996). Here we report the functional relationship between RNA processing (RNA editing and RNA cleavage) and translation using our chloroplast *in vitro* translation system. Chloroplast genes encoding the 9 kDa iron-sulfur protein of photosystem I (*psaC*) and the ND4 subunit of a putative NADH dehydrogenase complex (*ndhD*) are known to be organized as a cluster and to produce a dicistronic pre-mRNA which is then cleaved into a set of short mRNA species in higher plants (Hayashida *et al.*, 1987; Matsubayashi *et al.*, 1987; Schantz and Bogorad, 1988; Steppuhn *et al.*, 1989; Neckermann *et al.*, 1994). The ND4 subunits deduced from *ndhD*s of land plant chloroplasts and cyanobacteria show a high degree of overall sequence homology, while their N-termini are variable in length and amino acid composition. Sequence analysis of *ndhD* transcripts has shown that RNA editing of an internal ACG triplet in tobacco, spinach and snapdragon leads to the creation of an AUG codon, corresponding to the start codon in other plant species (Neckermann *et al.*, 1994). Our *in vitro* studies showed that both RNA editing and intercistronic cleavage of the *psaC*-*ndhD* pre-mRNA are prerequisites for translation of tobacco chloroplast *ndhD* mRNA. A molecular mechanism of the translational inhibition by the dicistronic pre-mRNA and its biological significance are discussed.

Results

Multiple 5' ends of *ndhD* mRNAs

The *ndhD* gene is located in the middle of the small single-copy region of tobacco chloroplast DNA (Shinozaki *et al.*, 1986). This gene has been shown to be co-transcribed with the upstream *psaC* and the resultant dicistronic pre-mRNA of ~2.5 kb has been processed into monocistronic forms of ~2 kb (*ndhD*) and ~0.5 kb (*psaC*) in tobacco chloroplasts (Hayashida *et al.*, 1987; Matsubayashi *et al.*, 1987). To determine precisely intercistronic processing sites in the *psaC*-*ndhD* pre-mRNA, an RNase protection assay was carried out using total RNA preparations from tobacco leaves and non-green cultured cells (Figure 1A). Two overlapping probes (I and II) were used to facilitate estimation of the 5' ends of *ndhD* mRNA and the 3' ends of *psaC* mRNA. As shown in Figure 1B, the unprocessed mRNA (probably pre-mRNA) was detected at a very low level and most of the transcripts were accumulated as they were processed and are probably monocistronic mRNAs both in leaves and cultured cells. A very low accumulation of the pre-mRNA was also shown by northern blot analysis (Hayashida *et al.*, 1987).

Six discrete bands representing *ndhD* mRNAs and four different bands representing *psaC* mRNAs were observed. The 5' end of processed *ndhD* mRNAs and the 3' ends of processed *psaC* mRNAs were estimated from these protected bands and mapped on the intercistronic region (Figure 1C). The most upstream 5' end (A) is located inside the *psaC* coding region, suggesting that the upstream mRNA fragment is not functional as *psaC* mRNA. The next site (B) is mapped at an U cluster (U₈) in front of a possible stem-loop structure, and the rest (C-F) are located

Fig. 1. Determination of intercistronic processing sites in the *psaC*-*ndhD* mRNA. (A) Strategy to detect ends of processed mRNAs by RNase protection assay. Two arrows indicates antisense RNA probes I (positions -118 to +185) and II (positions -239 to -1). A-F and a-d show 5' ends of processed *ndhD* mRNA and 3' ends of *psaC* mRNA, respectively. Examples of protected mRNA fragments (a and C) are shown below. (B) Gel patterns of protected fragments from RNA preparations hybridized with probe I or II. P, RNA probe only; I-IV, tobacco leaf stages I-IV (1-3 cm, 3-5 cm, 5-10 cm, 10-15 cm, respectively); Y, yeast RNA; D, dark adapted (48 h darkness at 28°C) stage I leaves; M, size marker (ψ x174 RF *Hinc*II digest). (C) Processing sites mapped on the intercistronic region. Horizontal arrows show an inverted repeat, underlines denote SD-like (GAG), AUG triplet, inhibitory element and editing codon.

within the stem-loop structure. Two of the 3' ends of *psaC* mRNAs are mapped at inside (a and b) and downstream (c and d) regions of the stem-loop structure. Among them, the positions of only two *ndhD* mRNA 5' ends (C and D) match those of *psaC* 3' ends (a and b), indicating that monocistronic mRNAs for *psaC* and *ndhD* are produced by multiple endonucleolytic cleavages or by an endonucleolytic cleavage followed by exonucleolytic trimming. *psaC* mRNA fragments and *ndhD* mRNA parts produced by cleavage at sites A, B, E and F, and at sites c and d, respectively, could not be detected, suggesting that these mRNA fragments are non-functional and hence unstable. We also examined processing patterns in green leaves of various sizes (Figure 1B) and non-green cultured cells (containing non-photosynthetic plastids, data not shown), and found that the pattern of the processed mRNA level is apparently constant.

Fig. 2. Determination of the initiation codon in the *ndhD* mRNA. (A) Schematic representation of *ndhD* mRNAs used for *in vitro* translation. The 5'-UTR sequence is shown in Figure 1C. 'D' stands for *ndhD* mRNA and 'ed' for mRNA with the edited AUG. (B) SDS-PAGE patterns of *in vitro* translation products. Size markers (kDa) are shown on both sides. The translation efficiency was calculated from the band intensity of three independent experiments (D1ed as 100%).

Only the edited AUG codon in *ndhD* mRNA functions as an initiation codon

The initiation codon of tobacco *ndhD* gene was previously believed to be the ATG located 95 bp downstream from the *psaC* coding region and a Shine-Dalgarno (SD)-like sequence (GAG) found 12 bp upstream from the ATG was assigned as a potential ribosome-binding site (see Figure 1C, Matsubayashi *et al.*, 1987). However, C to U editing has been shown to occur at ACG 25 nt downstream from the AUG in the tobacco *ndhD* transcript, which leads to the creation of an in-frame AUG codon (Neckermann *et al.*, 1994). In order to determine which AUG is the genuine initiation codon of *ndhD* mRNA, we assayed template activity of edited and unedited *ndhD* mRNAs using the chloroplast *in vitro* translation system (Hirose and Sugiura, 1996). PCR-amplified DNA fragments from edited (AUG) and unedited (ACG) monocistronic *ndhD* mRNA portions (positions -118 to +185, containing the intercistronic region and the first 12 % coding region) were fused to *lacZ* (positions +186 to +564). Fusion to *lacZ* mRNA was expected to make the synthesized product soluble and stable. The *ndhD-lacZ* mRNAs synthesized *in vitro* by T3 RNA polymerase (Figure 2A) were subjected to *in vitro* translation. As shown in Figure 2B, only the

edited mRNA (D1ed) produced a single product of the expected size (21 kDa), while the unedited mRNA (D1) produced none, in spite of the presence of an upstream AUG. Furthermore, mutation of the upstream AUG to UAC (D2, D2ed) did not lead to translation of the unedited mRNA but slightly increased translation of the edited mRNA. These results indicate that the only edited AUG codon acts as the actual initiation codon of tobacco *ndhD* mRNA, and that the upstream AUG has no function as an initiation codon, in spite of the preceding SD-like sequence (GAG).

The stem-loop structure located 3 nt upstream from the upstream AUG may prevent ribosomes from binding to this AUG. In order to examine this possibility, we synthesized *in vitro* shortened mRNAs (corresponding to F, position -53, in Figure 1C) with unedited ACG or edited AUG (D3 and D3ed in Figure 2A) and analyzed their translation *in vitro*. As shown in Figure 2B, the translation product of 21 kDa was detected only from the edited mRNA but not from the unedited mRNA, indicating that the upstream AUG does not function as an initiation codon even after removal of the stem-loop configuration. Furthermore, the result suggests that the upstream stem-loop region is not crucial for translation of edited *ndhD* mRNAs and that the shortest *ndhD* mRNA F found *in vivo* is functional.

Extent of RNA editing during development and light conditions

RNA editing at the ACG triplet in *ndhD* transcripts has been reported to be partial in three dicotyledonous plant chloroplasts (Neckermann *et al.*, 1994). We analyzed in more detail the extent of edited *ndhD* transcripts in tobacco leaves with four different sizes (stages I, II, III and IV), dark-adapted leaves (stage I), non-green cultured BY-2 cells, bleached seedlings grown on a medium containing streptomycin and spectinomycin (plastid translation is inhibited) and mutant green seedlings which are resistant to streptomycin and spectinomycin (*spc2*; Svab and Maliga, 1991). As shown in Figure 3, the extent of edited *ndhD* transcripts changed depending on the sizes of leaves, plastid types, light/dark conditions and plastid translation activity. It is the highest in young stage I (1–3 cm) leaves (56%); however, dark adaptation of stage I leaves led to the reduction of the extent of editing (34%). In non-photosynthetic plastids of bleached seedlings and of cultured cells, no editing was observed (<5%), although editing occurred at a low level (20%) in green (*spc2*) seedlings grown in the presence of these antibiotics. From these data, it is evident that the level of edited transcripts is higher in photosynthetically active tissues. On the other hand, editing of tobacco *atpA* transcripts (site I) was complete in all the above conditions [Figure 3 in Hirose *et al.* (1996)].

Intercistronic cleavage is required for translational control of *ndhD* mRNA

As mentioned earlier, most of the transcripts from the *psaC-ndhD* cluster are accumulated as processed/monocistronic mRNAs. In order to investigate whether cleavage of the *psaC-ndhD* pre-mRNA is required for *ndhD* translation, we synthesized dicistronic mRNAs from the *psaC-ndhD* genes as template (CD1 and CD1ed in Figure 4A). As shown in Figure 4B, no *ndhD* product could be detected

Fig. 3. Extent of ACG to AUG editing in *ndhD* transcripts from tobacco stage I, II, III and IV leaves, dark adapted stage I leaves, green SPC2 seedlings, bleached seedlings and cultured (BY-2) cells. The extent of the editing was estimated from the intensity of bands corresponding to C and U in cDNA sequencing ladders as shown in the insets. Ladders are in the order G, A, T, C from the left, and triangles indicate the editing site.

Fig. 4. Effect of intercistronic cleavage on translation of *ndhD* and *psaC* mRNAs. (A) Schematic representation of *psaC* and/or *ndhD* dicistronic and monocistronic mRNAs used for *in vitro* translation. Expected products are of 9 kDa (*psaC*) and 21 kDa (*ndhD-lacZ*). 'CD' stands for *psaC-ndhD* mRNA and 'C' for *psaC* mRNA. (B and C) SDS-PAGE patterns of *in vitro* translation products. Size markers (kDa) are shown on both sides. Translation efficiencies are shown below (*D1ed* and *C2* as 100%).

from the dicistronic mRNAs, even with the edited AUG under conditions such that the edited monocistronic *ndhD* mRNA produced 21 kDa product. This result indicates that the intercistronic cleavage is a critical step for translation of tobacco *ndhD* mRNA and suggests the

Fig. 5. Identification of inhibitory elements for translation of *ndhD* mRNA. (A) Schematic representation of a series of 5' deletion mRNAs. (B) Inhibitory elements are mapped on the *psaC-ndhD* mRNA. The 8 nt sequence in the 5' *psaC* coding region is complementary to that in front of the *ndhD* coding region. The former was mutated as in *CD1ed/m*. (C and D) SDS-PAGE patterns of *in vitro* translation products. Size markers are shown on the left. Translation efficiencies are shown below (*D1ed* as 100%).

existence of a translational inhibitor in the *psaC* mRNA part.

In order to identify the negative elements for *ndhD* translation from the dicistronic mRNA, a series of 5' deletion mRNAs were constructed as shown in Figure 5A. Their *in vitro* translation revealed that an *ndhD* mRNA (*CD4ed*) containing the 3' half (120 nt) of the *psaC* coding region could still be translated in the same way as the *D1ed* and *D3ed* mRNAs, while translation efficiency was reduced drastically with three other mRNAs (*CD1ed*, *CD2ed* and *CD3ed*) that contain the whole coding region of *psaC* (Figure 5C). This observation suggests that the negative element is located within the 5' *psaC* protein-coding region (positions +1 to +124) or its 5'-untranslated region (5'-UTR). Computer analysis showed that 8 nt in the 5' coding region (5'-AGUAAAGA-3', positions +12 to +19) can base-pair with a sequence in the *ndhD* 5'-UTR (5'-UCUUUACU-3', positions -8 to -1, see Figure 5B). These two complementary RNA sequences may inhibit translation by preventing ribosomes from binding to the 5'-UTR of *ndhD* mRNA.

In order to prove this hypothesis, we constructed a mutant *psaC-ndhD* mRNA (*CD1ed/m*) with a mutated 8 nt sequence (from AGUAAAGA to ACGUCUCA) so

Fig. 6. RNase T2 probing of the monocistronic (D1ed), dicistronic (CD1ed) and mutated dicistronic (CD1ed/m) mRNAs (see Figures 2A, 4A and 5B). The 8 nt sequence is boxed on the left. Unprotected nucleotides (dense bands) in the 8 nt sequence of D1 ed and CD1ed/m mRNAs are indicated by arrows.

as not to base-pair with the counterpart (Figure 5B). As shown in Figure 5D, the translation activity was restored in part by this mutation. Base-pairing of the 8 nt sequence pair was then confirmed by RNase T2 probing. The 8 nt sequence in the dicistronic mRNA (CD1ed) is protected by RNase T2 digestion (RNase T2 digests ssRNA but not dsRNA), while the same regions in the monocistronic mRNA (D1ed) and mutated dicistronic mRNA (CD1ed/m) are cleaved (Figure 6). These observations indicate that this 8 nt sequence is at least one of the negative elements for translation of the dicistronic *psaC*–*ndhD* mRNA.

As no *psaC* product was detected from these dicistronic mRNAs (see Figure 5C), we next analyzed the effect of RNA cleavage on translation of the *psaC* part. A pair of dicistronic (C1) and monocistronic (C2) mRNAs were constructed (Figure 4A) and subjected to *in vitro* translation. The dicistronic mRNA was truncated at position –22, in front of the 8 nt element to produce the monocistronic *psaC* mRNA. As shown in Figure 4C, the expected translation product of 9 kDa was detected from the monocistronic *psaC* mRNA (C2), but only slightly from the dicistronic mRNA (C1). This suggests that RNA cleavage is required for efficient translation of the upstream *psaC* mRNA as well as translation of the downstream *ndhD* mRNA.

Discussion

Most of the RNA editing events found in chloroplasts restore the conserved amino acids (e.g. Maier *et al.*, 1995) except for one silent editing (editing at the third base of a codon) in tobacco *atpA* transcripts (Hirose *et al.*, 1996). The creation of an AUG codon from a ACG triplet by RNA editing has been reported in transcripts from *rpl2* in maize (Hoch *et al.*, 1991), *psbL* in tobacco (Kudla *et al.*, 1992), bell pepper (Kuntz *et al.*, 1992) and spinach (Bock *et al.*, 1993), *ndhD* in three dicotyledonous plants (Neckermann *et al.*, 1994) and *petL* (*ycf7*) in black pine (Wakasugi *et al.*, 1996). However, there is no direct experimental evidence showing that the edited AUG is an actual initiation codon. In the case of tobacco *ndhD* transcripts, the edited AUG is located in the same reading frame nine codons after the AUG triplet that was initially proposed as the initiation codon for *ndhD*, and this upstream AUG is preceded by an SD-like sequence at the appropriate position (Matsubayashi *et al.*, 1987). Our

in vitro translation analysis showed clearly that only the edited AUG codon acts as an initiation codon and the upstream AUG has no function for the translational initiation. Neither SD-like nor translation promoting sequence (TPS)-like sequences (Thanaraj and Pandit, 1989) are found within the 27 nt upstream region from the functional initiation codon in the *ndhD* mRNA. Furthermore, sequences similar to *cis*-acting elements for tobacco *psbA* translation (Hirose and Sugiura, 1996) are not found in its 5'-UTR. These observations suggest that a novel type of ribosome-binding/translational initiation mechanism operates for the translation of *ndhD* mRNA. It is noteworthy that, despite the existence of an SD-like element, the upstream AUG is not functional. Moreover, mutation of the upstream AUG caused a slight increase in translation from the edited AUG, suggesting that the upstream AUG exerts a competitive effect upon ribosome binding. The possibility that a stem-loop structure present in front of the upstream AUG triplet prevents ribosomes from binding to the potential initiation site would be excluded by an experiment which showed that an mRNA lacking this structure is not functional either. However, this stem-loop structure may reduce the competitive effect by the upstream AUG, as translation decreased from the D3ed mRNA which lacks this structure (see Figure 2).

The RNA editing reaction has been shown to be a rapid post-transcriptional event which precedes RNA cleavage and splicing in chloroplasts (Freyer *et al.*, 1993; Hirose *et al.*, 1994; Ruf *et al.*, 1994). Most transcripts containing editing sites are known to be fully edited. However, a portion of *ndhD* mRNAs was shown to be unedited. The extent of editing within this site is variable, depending on developmental and light conditions, and apparently correlates with the photosynthetic activity of plastids. As the ACG to AUG editing is essential for translation, the synthesis of the *ndhD* product is controlled by the editing event, whose extent is differentially regulated by developmental and environmental conditions. Based on the observation that no editing was observed in tobacco seedlings grown in the presence of spectinomycin and streptomycin, this editing may require chloroplast translation products. Editing of *ndhD* mRNA has been reported to require a depletable *trans*-factor that is distinct from that required for *psbL* editing (Chaudhuri *et al.*, 1995; Chaudhuri and Maliga, 1996). It is likely that the level of such a depletable factor is controlled by various external and internal conditions.

Besides *ndhD* mRNA, the occurrence of the silent editing of tobacco *atpA* mRNA has been shown to depend on plastid stage and light conditions, and its extent is high in the dark-adapted leaves (Hirose *et al.*, 1996). Partial editing has been also observed in spinach *psbF* and *psbL* mRNAs from non-photosynthetic proplastids of seeds and from amyloplasts in roots, but no difference was observed between light and dark grown seedlings (Bock *et al.*, 1993). The above observations on differential editing suggest that site-specific factors, which are regulated by various conditions, are involved in editing within individual editing sites of some chloroplast transcripts. Our report provides experimental evidence for an additional function of editing as a regulatory step of protein synthesis in chloroplasts.

Chloroplast operons are generally composed of func-

Fig. 7. A model of differential synthesis of the 9 kDa iron-sulfur protein of photosystem I and the ND4 subunit of a NADH dehydrogenase from the *psaC-ndhD* gene cluster. After transcription from the gene cluster, the pre-mRNA forms a base-pair between a and b so that no translation occurs. Intercistronic cleavage is required for translation of both *psaC* and *ndhD* mRNAs. In addition, RNA editing is essential for *ndhD* translation.

tionally related genes (e.g. ribosomal protein operons; Tanaka *et al.*, 1986); however, several operons consisting of functionally unrelated genes have also been reported (e.g. the *psaA-psaB-rps14* operon; Meng *et al.*, 1988). The *psaC-ndhD* gene cluster consists of functionally unrelated genes encoding a photosystem I subunit and an NADH dehydrogenase subunit. We showed that the dicistronic *psaC-ndhD* mRNA produces no *ndhD* product, and that intercistronic cleavage is required for translation of the *ndhD* part. Based on the mutation experiments and enzymatic probing assay, we propose a model that an intramolecular interaction between a region immediately upstream from the edited AUG initiation codon of *ndhD* mRNA and a 5' part of *psaC* coding region in the *psaC-ndhD* mRNA inhibits translation of *ndhD* mRNA (Figure 7).

Efficient synthesis of the *psaC* protein is expected to require the intercistronic cleavage of this model, and this was confirmed by our *in vitro* analysis: the 9 kDa product was detected with a monocistronic *psaC* mRNA while none or a very low level was detected with dicistronic mRNAs. Our preliminary experiment indicated that a portion of the unprocessed (probably dicistronic) mRNA is present in >70S fractions *in vivo*, suggesting that ribosomes can bind to the 5'-UTR of *psaC-ndhD* dicistronic mRNA and initiate translation, but pause at the site of the intramolecular interaction. A minor fraction of the bound ribosomes may read through the above site to produce the *psaC* protein from the dicistronic mRNA (C1) but not from another dicistronic mRNA (CD1ed). Read-through might occur, depending on the overall configurations of dicistronic mRNAs.

Generally, photosystem components including the *psaC* protein must be highly synthesized in chloroplasts of light-grown plants. On the other hand, translation products

from *ndh* genes are hardly detectable in higher plant chloroplasts, and detection of the *ndhD* protein has not been reported, suggesting that synthesis of *ndh* proteins is very low in chloroplasts. The differential synthesis of *psaC* and *ndhD* products can be explained at least in part by partial RNA editing of the *ndhD* initiation codon, with control at the level of RNA processing (Figure 7). At present we do not know whether the partial RNA editing is the sole control factor. Translational control cannot be excluded, for example, different mRNA template activity or different initiation frequencies between *psaC* and *ndhD* monocistronic mRNAs. In order to examine this activity, one should normalize the effects of translational elongation, 3'-UTR and product stability (e.g. using mRNAs fused to *lacZ* mRNA; Hirose and Sugiura, 1996). An additional interesting issue to be analyzed is the exact order of RNA cleavage and RNA editing or the relationship between these two steps. Chloroplast *in vitro* systems supporting RNA editing would be very desirable.

Materials and methods

Plant materials

Tobacco, *Nicotiana tabacum* var. Blight Yellow 4, was grown in a growth chamber (28°C, 16 h light and 8 h dark) for 2 (stage I), 3 (stage II), 4 (stage III) and 5 (stage IV) weeks. Dark-adapted stage I plants, wild type and *spc2* mutant seedlings were obtained as described (Hirose *et al.*, 1996). Tobacco cultured cells (line BY-2) were grown in Murashige-Skoog medium containing 3% sucrose and 0.1 µg/ml 2,4-D for 3.5 days.

Analysis of *in vivo* transcripts

Preparation of total RNAs and an RNase protection assay were carried out as described (Hirose *et al.*, 1993). Portions of the *psaC-ndhD* genes (positions -118 to +185 for probe I, positions -239 to -1 for probe II) were cloned into pBluescriptII SK⁺ and digested with *Bam*HI. ³²P-labeled antisense RNA probes I and II were synthesized by using an *In vitro* transcription kit (Stratagene). Direct DNA sequencing of PCR-amplified cDNA fragments was carried out as described (Hirose *et al.*, 1996).

Plasmid constructions

cDNA was synthesized from 1 µg of total RNA of stage I leaves according to the instruction manual (Promega). *ndhD* portions with ATG and ACG at position +1 were amplified by PCR from the cDNA and tobacco chloroplast DNA, respectively (Figure 2A). The amplified DNA fragments were digested with *Eco*RI and *Bam*HI and cloned into pBluescriptII SK⁺, by which the *ndhD* regions (positions -118 to +185) is fused in-frame to the 3' *lacZ* coding region (+186 to +564). 5' deletion constructs of *psaC/ndhD* were made by inverse PCR using appropriate primer pairs and construct CD1ed as template. Site-directed mutagenesis was performed using Transformer™ Site Directed Mutagenesis kit Version 2 (Clontech) using primer pairs and constructs D1ed and CD1ed as templates. Plasmid constructs were amplified in *Escherichia coli* XL-1 Blue, isolated by the alkaline lysis method and purified further by CsCl gradient centrifugation. Plasmid DNAs were linearized with *Nae*I, treated with phenol/chloroform and precipitated with ethanol. mRNAs were synthesized by T3 RNA polymerase using T3 MEGASCRIP (Ambion), and include a 56 nt vector sequence in their 5' ends (not shown).

Preparation of chloroplast S30 fraction and *in vitro* translation

Preparation of S30 fractions from intact tobacco chloroplasts and *in vitro* translation reaction were carried out essentially as described (Hirose and Sugiura, 1996). The dialyzed S30 fraction (~500 µl, ~15 mg/ml protein) could be stored at -70°C for up to 6 months. The *in vitro* translation reaction was carried out at 30°C for 30 min under conditions optimized with *ndhD* mRNAs and the synthesized products were separated by SDS-PAGE. Protein size markers used were from Rainbow marker

(Amersham). The separated products were visualized and quantified by a Bio-imaging Analyzer BAS2000 (Fuji Photo Film Co.).

Enzymatic probing of RNA structure and primer extension

mRNAs were synthesized from constructs CD1ed, D1ed and CD1ed/m, each of which was linearized with *Bss*HII followed by purification in denaturing gel electrophoresis. RNA samples (10 pmol each) were preincubated for 20 min at 25°C in 30 mM HEPES-KOH (pH 7.7), 60 mM K-acetate, 10 mM Mg-acetate and 60 mM NH₄Cl, and digested with 0.1 U RNase T2 (Sigma) for 5 min at 25°C. Digested RNA samples were extracted by phenol/chloroform and precipitated with ethanol. The primer (positions 34–17 in the *ndhD* coding region), labeled at its 5' end with T4 polynucleotide kinase and γ^{32} P-ATP, was annealed to digested RNAs at 95°C for 1 min and this was followed by cooling down to 42°C. Reverse transcription was carried out by using a cDNA cycle kit (In Vitrogen). Primer extension samples were separated by 10% PAGE containing 7 M urea and visualized by a Bio-imaging Analyzer BAS2000.

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