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RNA processing alters open reading frame stoichiometry from the large ATP synthase gene cluster of spinach chloroplasts

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Abstract

The large ATP synthase gene cluster of spinach chloroplasts is a multigenic cluster that encodes the small ribosomal subunit 2 followed by four ATP synthase subunits. The stoichiometry of the ATP synthase gene products from this cluster changes markedly between transcription and assembly of the complex. The two primary transcripts from this gene cluster undergo a complex series of RNA processing steps. Here we show that the extensive RNA processing that the large ATP synthase gene cluster transcripts undergo results in a substantial change in the stoichiometry of complete open reading frames (ORFs) of the four ATP synthase genes. Processing directly affects the stoichiometry of open reading frames from this gene cluster by intragenic cleavage. It may also affect open reading frame stoichiometry more indirectly, but equally significantly, by cleavage-induced alteration of stability of some of the processed transcripts relative to the others.

Introduction

All of the polypeptide complexes embedded in chloroplast thylakoid membranes are derived from a combination of plastid and nuclear-encoded subunits [7]. Efficient biogenesis of chloroplast complexes requires precise coordination of chloroplast and nuclear gene expression to produce polypeptides in the correct stoichiometries [12, 19, 21, 23, 27].

Mechanisms by which plant cells coordinately modulate nuclear and chloroplast gene expression have been intensely studied [12, 19, 21, 23, 27]. The expression of nuclear-encoded chloroplast genes is primarily regulated at the transcriptional level in response to light [37]. In contrast, transcription of most chloroplast-encoded genes is essentially constitutive, with global RNA fluctuations depending on the developmental stage of the chloroplast [5, 11, 12, 13, 23]. Post-transcriptional regulation provides fundamental control for most plastid gene expression.

Many chloroplast-encoded genes are arranged in multigenic clusters reminiscent of bacterial genomes. These clusters often encode genes required for more than one chloroplast complex [13, 21, 25, 27, 31, 32]. Our research concentrates on the large ATP synthase gene cluster from spinach chloroplasts. This cluster encodes (in order) the small ribosomal subunit protein 2 (*rps*2), and four ATP synthase subunits: CF_0-IV (*atp*I), CF_0-III (*atp*H), CF_0-I (*atpF*) and CF_1-α (*atpA*) (Fig. 1) ([18] for nomenclature [15]). The transcripts of this cluster display a complex pattern of RNA maturation [18, 34]. The two primary transcripts originate upstream of *rps2* or *atpH* and terminate downstream of *atpA*. This results in an initial RNA ratio of X:1:1:1 for the four ATP synthase gene products (*atpI*:*atpH*:*atpF*:*atpA*), with the value of X depending on the relative strengths of the two promoters [34]. After processing, there are more than thirty RNA species, all of which appear to be bound by ribosomes (N. Stollar and M.J. Hollingsworth, unpublished). The polypeptides encoded by the large ATP synthase gene cluster accumulate in a ratio of 1:5–12:1:3 in the mature complex [22, 36]. No combination of transcription from the two promoters could yield a ratio of RNA for the four subunits proportional to the final stoichiometry

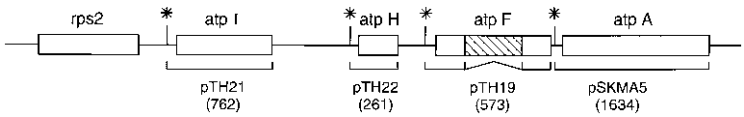

Figure 1. Organization of the spinach plastid large ATP synthase gene cluster. Transcription is from left to right and begins upstream from *rps2* and upstream from *atpH*. Genes are shown as open boxes. The group II intron interrupting *atpF* is represented by a shadowed box. Asterisks indicate the location of putative ribosome-binding sites [3]. Regions from the cluster that were cloned and used to generate probes are diagrammed as lines below the cluster. Clone names and the length of the chloroplast sequences contained within them (in nt) are noted below the lines.

of polypeptides. Thus the expression of this cluster is clearly under post-transcriptional control.

Other multigenic clusters from land plant chloroplasts display a complex RNA maturation pattern similar to that observed for the large ATP synthase gene cluster [13, 23]. This extensive and specific RNA processing is presumably costly in terms of energy to the plant, but the function of processing in chloroplast gene expression is undefined. It is likely that RNA processing affects the translational efficiency or stability of particular messages by altering regulatory regions hypothesized to interact with transacting factors [14, 10]. Cleavages also disrupt open reading frames (ORFs), with the result that only a subset of the ORFs encoded on a multigenic transcript are potentially translation-competent.

Here we present evidence that the extensive RNA processing the large ATP synthase gene cluster transcripts undergo results in a marked change in the stoichiometry of complete ORFs of the four ATP synthase genes. Using an RNAse protection assay to quantify the ratios of the four ATP synthase ORFs, we demonstrate that processing results in an accumulation of complete (and therefore potentially translation-competent) ATP synthase ORFs in a 2:11:3:1 ratio. This stoichiometry is significantly different from the initial transcription ratio of X:1:1:1 and much closer to the 1:5–12:1:3 stoichiometry of polypeptides in the mature ATP synthase complex.

Materials and methods

Plant material

Spinach (*Spinacia oleracea*, winter Bloomsdale) seeds were germinated in vermiculite and then transferred to hydroponic tanks. Young healthy leaves ca. 3 cm long were used for this study.

Chloroplast isolation

Chloroplasts were isolated on Percoll step gradients as described by Orozco *et al.* [24]. Intact plastids were aliquoted, frozen on dry ice, and stored at $-75\,^{\circ}$C.

RNA isolation

Nucleic acids were isolated from frozen chloroplasts by phenol extraction in the presence of guanidine as described previously [11]. DNA was removed by incubating the isolated nucleic acids with RNAse-free DNAse (Promega), followed by two phenol/chloroform extractions and ethanol precipitation. The RNAs were resuspended in diethyl pyrocarbonate-treated water and the concentration determined by spectrophotometry.

Clones and probes

Plasmids containing the complete ORF with ribosome-binding site (RBS) were constructed for *atpI*, *atpH* and *atpF*. Inserts containing the appropriate sequences were generated via polymerase chain reaction (PCR) with oligonucleotides of the following sequences:
atpI: 5′-oligo:
TGTAAGAGGACAATATGAAT (1419–1438)
3′-oligo: AAATCAATGATGACCTTCCAGGGA (2180–2157).
atpH: 5′-oligo:
GTGTGTGAGGAACTTTATCATG (2849–2872)
3′-oligo: GGATTAAACAAAAGGATTCGCAAAT (3094–3118).
atpF: 5′-oligo:
TTATCTATAAGAGGAGATCA (3490–3509).
3′-oligo: ATCAGTTATTTCGTTCATCGCACCA (4802–4826).

Numbering is according to Hudson *et al.* [18]. Template DNA for the PCR reactions for *atpI* and *atpH* was pJB6, which contains the entire large ATP syn-

thase gene cluster [35]. The template for *atpF* was pT7BlueFsp2R, which contains the entire spliced *atpF* gene and 5′-untranslated region (5′ UTR). The PCR products were cloned into the vector pCR II (Invitrogen) between flanking *Eco*RI sites. These plasmids proved unstable over time and the *Eco*RI fragments were subcloned into *Eco*RI-digested Bluescript sk+ (Stratagene). The *atpI*, *atpH* and *atpF* clones are designated, respectively, pTH21, pTH22 and pTH19. The atpA ORF/RBS was derived from pSKMA5, encompassing bases 4790–6423. Figure 1 is a map of the ATP synthase gene cluster and designates the sequences contained in each of these plasmids.

Radiolabeled RNA probes were generated in standard *in vitro* transcription reactions such that the strand complementary to the mRNA was uniformly radiolabeled with ^{32}P-UTP [34]. These reactions utilized linearized pTH21, pTH22, pTH19, and pSKMA5 as template DNA. The RNA probes contain 54 (*atpA*), 69 (*atpH*), 80 (*atpI*) or 90 (*atpF*) bases of vector sequence in addition to the complement of the RBS/ORF.

RNAse protection assay

Assays were performed using the RPA II kit according to the manufacturer's specifications (Ambion). Briefly, 1–10 μg of total chloroplast RNA was precipitated with 10–500 pmol of specific RNA probe (100 000–500 000 cpm), hybridized overnight at 44 °C, digested with RNAse solution, and ethanol precipitated. The protected fragments were subjected to electrophoresis in 5% polyacrylamide/7 M urea, followed by autoradiography. The inclusion of 54–90 bases of vector sequence at the 5′ end of each RNA probe provided an internal control for RNAse activity. RNase protection assays of the probes in the presence of 10 μg nonspecific (yeast, Boehringer Mannheim) RNA resulted in complete digestion of the probes (data not shown). Individual bands representing full-length RNAs were excised and quantitated by liquid scintillation counting.

Results

Accumulation of full-length ATPase ORFs

Results from a typical RNAse protection experiment are shown in Fig. 2. Each of the probes used in these experiments is complementary to the complete ORF and potential ribosome binding site of one of the ATP synthase genes (Fig. 1). The actual ribosome binding sites are unknown, but potential sites identified by Bonham-Smith and Bourque were used in these experiments [3]. These probes will detect all transcripts containing, at a minimum, the RBS/ORF combination. Therefore, only potentially translation-competent RNAs from the total mRNA population are quantitated. As mentioned above, these RNA probes contain 54–90 bases of vector-derived sequence. This provides an internal control for RNAse digestion, since there will be a difference in mobility between undigested RNA probe and chloroplast RNA-protected probe (Fig. 2A). In control experiments, RNA probes were subjected to the RNase protection protocols in the presence of excess (10 μg) yeast RNA. No protection was observed in the absence of chloroplast RNA (data not shown).

The hybridization reactions were chloroplast RNA driven. Separate hybridizations over a range of 1–25 μg of chloroplast RNA resulted in a linear and proportional change in the amount of RNAse-resistant radiolabel (Fig. 2B).

The protected fragments corresponding to a full length ORF/RBS detected for each of the four ATP synthase genes were excised and quantitated by liquid scintillation counting (LSC). The values obtained by LSC were converted to molar amounts of each transcript. To obtain the stoichiometry of the four RNAs, the quantity of each protected transcript was normalized to that of *atpA*, which is the least abundant of the four. The quantitation of eleven experiments is summarized in the histogram in Fig. 3. Full-length ORFs for the four messages are present in a steady-state ratio of 2:11:3:1 (*atpI*:*atpH*:*atpF*:*atpA*).

Accumulation of shorter RNA products

Other abundant RNAs were detected in this assay. The strongest signals corresponded to an ca. 410 base fragment of *atpF* and two fragments of atpI of approximately 530 and 240 bases (Fig. 2A). A previously identified internal cleavage of *atpI* (+239) would generate the less-than-full-length fragments for this ORF [35]. The smaller *atpF* fragment corresponds to its 3′ exon.

Discussion

RNA processing appears to be involved in the expression of most, if not all, chloroplast genes. The actual

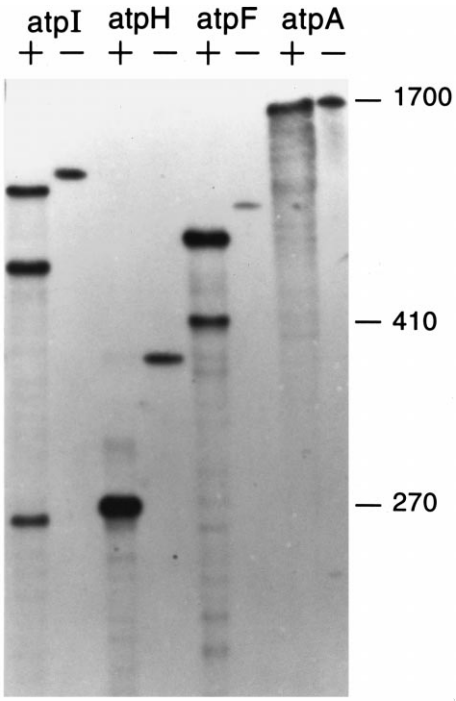

Figure 2. **A** (left). Relative transcript abundance of ATP synthase RBS/ORFs. Ten micrograms of total chloroplast RNA were hybridized to probes (200 pmol) complementary to the complete RBS/ORF sequence of each of the four ATP synthase genes. RNase-protected fragments were analyzed by gel electrophoresis and autoradiography. The name of each gene is indicated above the autoradiogram. Two samples for each gene were analyzed. Lanes designated + were digested with RNase A/T1. Lanes designates − were incubated as in the + lanes but without RNase. To avoid overexposure only 1/10 of the undigested controls were loaded. The slowest migrating fragment in each + lane corresponds to the length of the complete RBS/ORF sequence. The radioactivity in the bands was subsequently quantified as described in Materials and methods and used to determine the relative molar amounts of each ATP synthase RBS/ORF. (The *atpA* digestion products were typically subjected to longer electrophoresis than depicted here to ensure good separation between the fully and partially protected probe.) **B** (right). RNase protection as a function of chloroplast RNA quantity. One, 5, 10 or 25 μg of total chloroplast RNAs were subjected to RNase protection analysis in the presence of 500 pmol *atpH* probe. Each analysis was normalized to the protection detected for 1 μg of chloroplast RNA. Results from five analyses were combined to generate this graph. Vertical lines denote standard error.

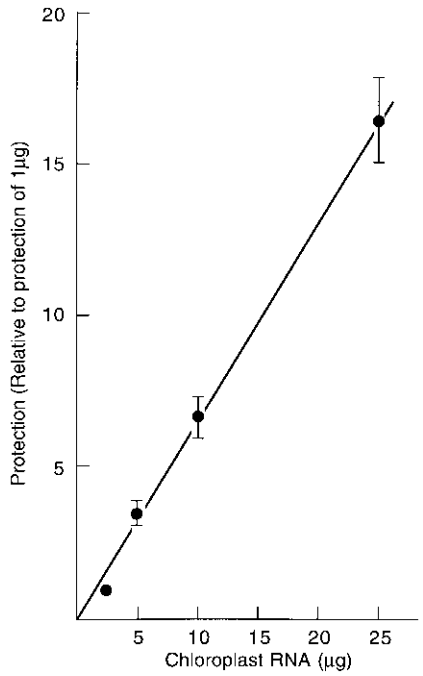

Figure 2. Continued.

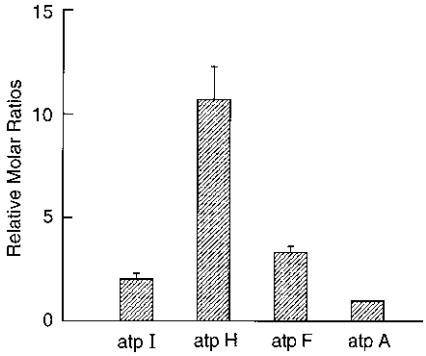

Figure 3. Relative molar ratios of RBS/ORFs from the large ATP synthase gene cluster. The results of 11 RNAse protection assays were averaged to generate the histogram. Quantitation of molar amounts of RNA is described in Materials and methods. As *atpA* is the least abundant RBS/ORF, abundance of the other three genes is reported relative to *atpA*. Vertical bars denote standard error.

effect of processing on a transcript will depend upon the location of the cleavage site. The most obvious effect of RNA processing is ORF disruption, through intragenic cleavage. Intergenic processing would have less direct, but potentially equally significant, effects on expression, depending upon the processing site. Structures in the 3′-untranslated regions (3′UTRs) of chloroplast transcripts have been shown to confer stability on the upstream transcript [17, 30]. Processing that resulted in alteration of such structures would be expected to change the stability of the processed transcript. Secondary structures in 5′-untranslated regions (5′UTRs) have also been proposed to affect RNA stability, but have been shown to primarily affect translation of the downstream ORF [6, 9, 10, 14, 17, 20, 27–29, 33, 38]. Thus processing that affects 5′UTR structure may have profound effects on the translation of the down-

stream ORF, as well as potentially having an effect on stability.

In addition to processing and its effects on stability and translation, there are several other posttranscriptional mechanisms that may affect the expression of the large ATP synthase gene cluster. Splicing of the group II intron in *atpF* is essential prior to translation of that ORF. We have also shown that ribosomes pause at several distinct sites during the translation of ORFs from this gene cluster [35]. Extensive pausing could result in alteration of translation efficiency of one ORF relative to another. Polypeptide turnover is an additional mechanism that affects the abundance of many chloroplast polypeptides [12].

Here we have shown that RNA processing markedly alters the stoichiometry of large ATP synthase gene cluster ORFs from the initial X:1:1:1 to 3:11:2:1. The processed ORF stoichiometry is distinctly more similar to the polypeptide ratio (1:5–12:1:3) than is the primary transcript stoichiometry. Therefore processing, through some combination of its effects on ORF disruption and transcript stability, markedly alters the abundance of complete ORFs from this gene cluster. Similar mechanisms resulting in a net change in the steady-state ratios of ORFs may be involved in the expression of other chloroplast gene clusters, the majority of which also exhibit an unbalanced stoichiometry between primary transcripts and polypeptide products [25, 31].

Although processing results in an ORF stoichiometry much closer to that of the ATP synthase polypeptides than the primary transcripts, this does not imply that ORF stoichiometry is directly proportional to ORF translation. In particular, the numerous endonucleolytic cleavages in 5′UTRs may result in an alteration of potential secondary structures in these regions, which could in turn affect the translational efficiency of the downstream ORF. An example of processing affecting translation is seen in the rbcL transcript in barley chloroplasts. When leaf segments are exposed to the plant hormone methyl jasmonate, the length of the *rbcL* 5′UTR increases, apparently due to alternative processing, and the translational efficiency of the new transcript is diminished [26]. In maize chloroplasts, it has been shown that translation of ORFs within multigenic chloroplast transcripts is transcript-context dependent [1, 2, 8]. Thus, RNA processing in the chloroplast not only alters the number of ORFs to be translated, but also provides several translational contexts for each ORF. This provides an opportunity to specifically regulate a single ORF from several perspectives. Experiments are currently in progress to investigate the effects of processing on translation efficiency of the ATP synthase ORFs.

Any or all of the possible outcomes of endonucleolytic cleavages discussed above would ultimately result in an alteration of the stoichiometry between primary transcripts and the polypeptides they encode. We speculate that the many transcripts of the large ATP synthase gene cluster generated by RNA processing may be both individually and globally regulated, with individual regulation to control subunit stoichiometry and global regulation to adjust the overall abundance of the ATP synthase complex in response to environmental and developmental conditions. Experiments are currently underway to determine the effects of RNA processing on the interaction of transcripts from the large ATP synthase gene cluster with *trans*-acting factors that might be expected to mediate such regulation.

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