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3′-Inverted repeats in plant mitochondrial mRNAs are processing signals rather than transcription terminators

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A number of mRNAs in plant mitochondria contain inverted repeats at their 3′-termini. These have been discussed as potential transcription terminators or, alternatively, as post-transcriptional processing and stability signals of longer precursor RNAs. *In vitro* transcription in a pea mitochondrial lysate now shows that transcription proceeds almost unimpeded through these inverted repeat structures. To investigate their potential function in mRNA processing, we developed an *in vitro* processing system from pea mitochondria. This *in vitro* system correctly processes synthetic precursor mRNAs containing the pea *atp9* double stem–loop structure, yielding the same 3′-termini observed *in vivo*. Analysis of the *in vitro*-generated products and of the processivity of the reaction suggests exonucleolytic degradation up to the stem–loop. The inverted repeat structures found at the 3′-termini of mRNAs in plant mitochondria are thus recognized as processing and most likely also stabilizing signals in transcript maturation, but do not terminate transcription.

Keywords: inverted repeat/*in vitro* processing/pea *atp9*/plant mitochondria

Introduction

In all organisms gene expression is regulated at many different levels. The amount of available RNA molecules is often controlled by a combination of transcriptional and post-transcriptional processes. RNA stability and RNA processing can regulate mRNA quantities corresponding to the requirements. The specific modes and pathways governing mRNA maturation and stability have been analyzed in bacteria and in the cytoplasm of eukaryotes (Birnstiel *et al.*, 1985; Manley and Proudfoot, 1994). Several studies have also investigated the control of mRNA levels in the organelles of eukaryotic cells, particularly in animal and yeast mitochondria and in chloroplasts of *Chlamydomonas* and higher plants (Rochaix, 1996; Sugita and Sugiura, 1996).

In plant mitochondria, however, little is known about the regulation of mRNA maturation and quantity control. Differential and specific RNA stabilities have been observed in plant mitochondria, suggesting controlled regulation of steady-state transcript half-lives (Finnegan and Brown, 1990; Mulligan *et al.*, 1991; Muise and Hauswirth, 1992; Binder *et al.*, 1996). Candidate structures for modulation of RNase access to mRNAs are inverted repeat (IR) sequence elements often found in the 3′-untranslated regions of mRNAs (Schuster *et al.*, 1986). The potential link between regulated RNA levels and the role of these IRs at the mRNA 3′-termini is as yet unresolved.

3′-IR structures are not necessarily associated with post-transcriptional RNA regulation, since such structures in the last nucleotides of the steady-state mRNAs have been identified as functioning as transcription terminators in bacteria and as regulating termination of transcription in animal mitochondria (Clayton, 1984; Cheng *et al.*, 1991; Cohen, 1995; Nudler *et al.*, 1995). In the latter genetic system, high expression of the rRNA genes is ensured by a modulating transcription termination signal at the 3′-terminus of the large rRNA. Here transcription is allowed to proceed to the downstream protein-coding genes only in the absence of transcription-terminating proteins (Christianson and Clayton, 1988; Daga *et al.*, 1993; Min and Zassenhaus, 1993; Fernandez-Silva *et al.*, 1997).

On the other hand, such stem–loop structures have been found to act as processing signals and stabilizing elements for the matured RNA in both bacterial and chloroplast transcripts (Stern and Gruissem, 1987; Manley and Proudfoot, 1994; Cohen, 1995; Rochaix, 1996; Sugita and Sugiura, 1996). Protein complexes binding to these signal structures in bacteria as well as in plastids contain a complex combination of exo- and endonucleases contributing to and effecting controlled maturation of the mRNAs (Stern and Gruissem, 1987; Hsu-Ching and Stern, 1991; Nickelsen and Link, 1993; Manley and Proudfoot, 1994; Cohen, 1995; Drager *et al*., 1996; Rochaix, 1996; Sugita and Sugiura, 1996). Binding efficiences of these mRNA-specific protein complexes furthermore determine the half-life and turnover of the given message. Upon disturbance or dissociation of the complex, the mRNA becomes accessible to degradation by nucleases.

In chloroplasts, several components of these activities have been identified and are found to be homologous, to a considerable extent, to the respective bacterial proteins (Carpousis *et al.*, 1994; Hayes *et al.*, 1996). This suggests that the framework controlling 3′-mRNA maturation pathways has been introduced into plastids by the ancestral cyanobacteria-like endosymbiont. In addition, genes for a number of participating RNA-binding proteins have been identified in the nuclear genome, which appear to be members of larger RNA-binding protein families and may be of a different, non-endosymbiont origin (Hayes *et al.*, 1996; Sugita and Sugiura, 1996).

In plant mitochondria, the IR structures identified at the 3′-termini of several mRNAs may have functions analogous to those seen in plastids, but may also serve entirely different functions (Schuster *et al.*, 1986). The

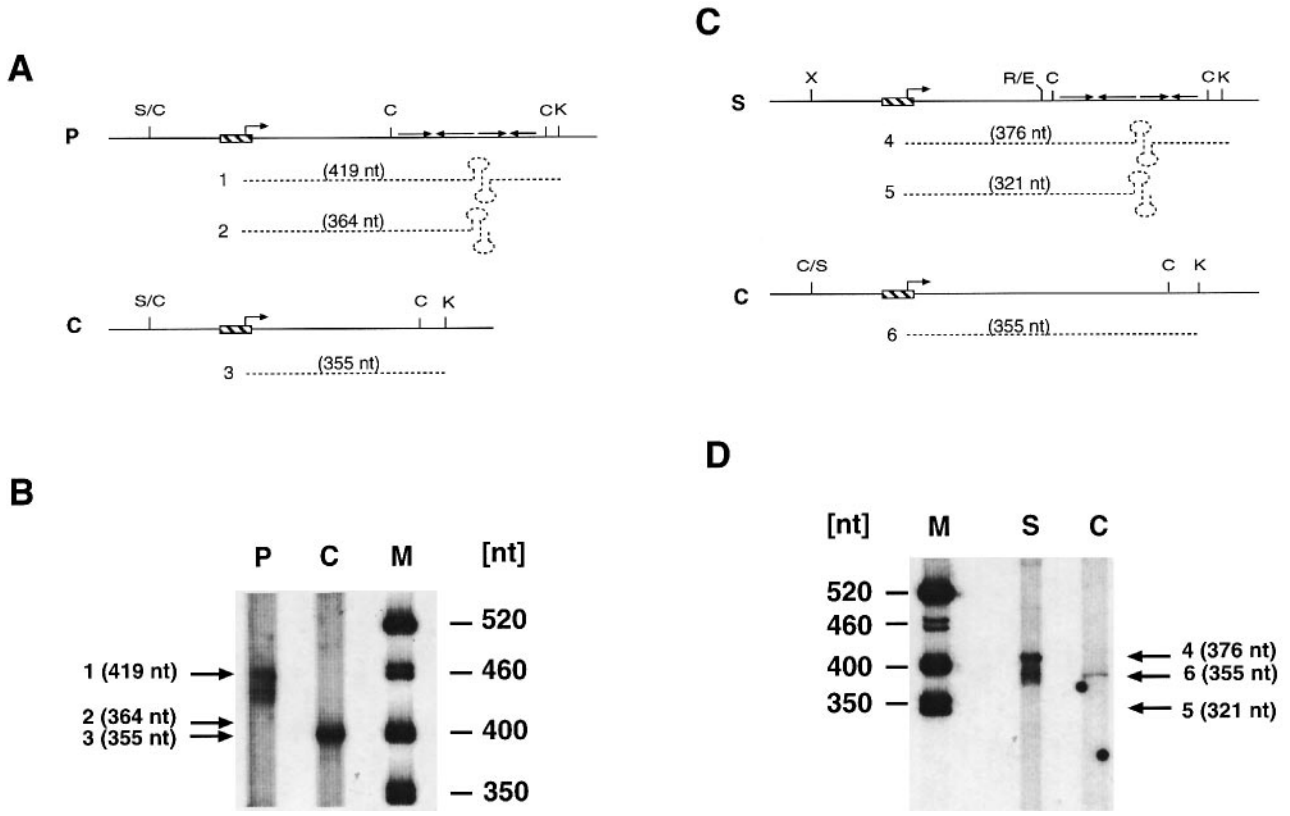

Fig. 1. Inverted repeat structures do not terminate transcription in plant mitochondria. (**A**) DNA templates were constructed with the pea *atp9* IR downstream of the pea *atp9* promoter (construct P). As control (construct C; patp9SK630), the *atp9* promoter without IR sequence was used. Structures and sizes of the expected run-off transcript (1), the expected termination product (2) and the control (3) are shown by dotted lines. (**B**) Incubation of these sequences in the *in vitro* transcription lysate yields the correct run-off transcript in the control as well as from the IR template P. Shortened RNA molecules contain 3′-ends scattered between the run-off length and the IR. (**C**) The influence of the surrounding sequence context was probed by embedding the pea IR into an *atp9* soybean promoter sequence context (construct S). Structures and sizes of the expected run-off transcript (4), the expected termination product (5) and the control (6) are shown by dotted lines. (**D**) RNA products obtained upon *in vitro* transcription from the soybean promoter with the pea protein lysate likewise show little termination of transcription at the IR. Restriction sites are indicated: *Sfu*I (S), *Cla*I (C), *Kpn*I (K), *Rsa*I (R), *Eco*RV (E) and *Xba*I (X). The DNA length standard (M) separates into the two strands in this denaturing gel and only gives approximate sizes for the RNA molecules, which are indicated with their correct sizes (arrows).

presence or absence of such IRs in otherwise similar transcription units has been correlated with differential steady-state abundance of mRNAs. In certain rice and wheat lines, for example, out of two cytochrome *b* genes only the gene containing a double stem–loop structure yields detectable amounts of transcript (Saalaoui *et al.*, 1990; Kaleikau *et al.*, 1992). These observations suggest a function for these IR structures in the control of RNA processing and stability in plant mitochondria.

We have now investigated the potential role of these stem–loop structures in plant mitochondria. Two different pea *in vitro* systems allowed differentiation between their possible regulatory function in transcription termination and/or mRNA processing. While transcription does not appear to be inhibited by these structures in a pea mitochondrial *in vitro* transcription system, a more complex protein lysate used in *in vitro* processing recognizes the IR structures as processing signals. The precursor mRNAs are trimmed correctly, yielding a stable RNA terminating just downstream of the IR structure. The complementary analysis of *in vivo* and *in vitro* processing at IRs in *Brassica* mitochondria described in the accompanying manuscript confirms the generality of these processing signals.

Results

The 3′-terminal IR does not terminate transcription in vitro

To investigate the potential function of the 3′-terminal IR in plant mitochondrial transcription termination, templates were constructed containing the pea mitochondrial *atp9* stem–loop region 294 nucleotides downstream of the corresponding pea *atp9* promoter (Figure 1). The *atp9* IR region has been found at the 3′-ends of all three transcripts of this gene with different 5′-extensions (Morikami and Nakamura, 1993). The *Kpn*I-linearized DNA template continues a further 55 nucleotides beyond the IR structure to monitor its influence. Run-off transcripts initiated at the *atp9* mitochondrial promoter in this template should thus cover 419 nucleotides, whereas transcripts terminated just downstream of the IR should be ~364 nucleotides in length.

When this construct is assayed in a pea *in vitro* transcription system (Binder *et al.*, 1995), transcription is initiated correctly at the *atp9* promoter (Figure 1). The vast majority of the RNA products correspond to run-off transcripts of 419 nucleotides containing the IR element. A portion of the transcripts generated on this IR template

accumulate as smaller RNA molecules, with lengths suggesting 3′-termini located between the IR and the ends of the run-off molecules. In the control reaction, a template without any IR yields only the full-length run-off transcripts, as indicated by the discrete RNA population (Figure 1B and D, lanes C).

To differentiate between the influences of upstream sequence and the IR structure, the pea *atp9* hairpin sequence was embedded into a heterologous nucleotide context downstream of the soybean mitochondrial *atp9* promoter (Figure 1C and D). The observed *in vitro* transcription products are again predominantly run-off RNAs (376 nucleotides) but, as in the pea context, smaller RNA species of ~321 nucleotides are generated with 3′-termini scattered between the IR structure and the run-off 3′-end. These observations show that the RNA sequence region 5′ to the IR has no detectable influence upon recognition and function of the IR. Consequently, 5′-shortened substrates were used for improved accuracy in the subsequent *in vitro* processing experiments, as detailed in Materials and methods.

The IR structure controls transcript processing

The above results suggest that in the *in vitro* transcription system, the presence of an IR structure stimulates the generation of RNA molecules smaller than the run-off products. If these smaller RNAs are derived by degradation of larger molecules, they should also be generated when synthetic RNA molecules containing the pea *atp9* IR are incubated with the transcriptionally active lysates.

To test this, RNA molecules containing 21 nucleotides upstream and 28 nucleotides downstream of the 47 nucleotide long IR were synthesized from T7 promoters and incubated in the mitochondrial *in vitro* transcription lysate. The added RNA molecules are found to be shortened similarly to the transcripts generated from the internal promoter in the mitochondrial *in vitro* transcription assay (Figure 2).

The presence of the IR structure thus appears to induce destabilization of the downstream RNA region, probably by initiating access to RNase(s), and at the same time to inhibit processivity of the RNase(s) at the IR. This conclusion is confirmed by the observation that RNA substrates without an IR are either stable or are degraded rapidly without enhanced stabilization of intermediate breakdown products becoming apparent (Figure 1B and D, lanes C).

However, the IR-induced RNA termini appear to be generated rather inefficiently in this mitochondrial subfraction optimized for *in vitro* transcription, suggesting that this lysate is somewhat depleted for one or more compounds essential for optimal mRNA maturation.

IR structures are also recognized in antisense RNAs

To differentiate between the influence of the structure of the IR and its primary sequence upon RNA processing, the fate of transcripts containing the pea *atp9* IR in antisense orientation was investigated (Figure 3). The IR element was inverted downstream of both sequence contexts, i.e. the pea and the soybean mitochondrial *atp9* promoter regions (Figure 1). *In vitro* transcription proceeds through the inverted IR sequence as little inhibited as

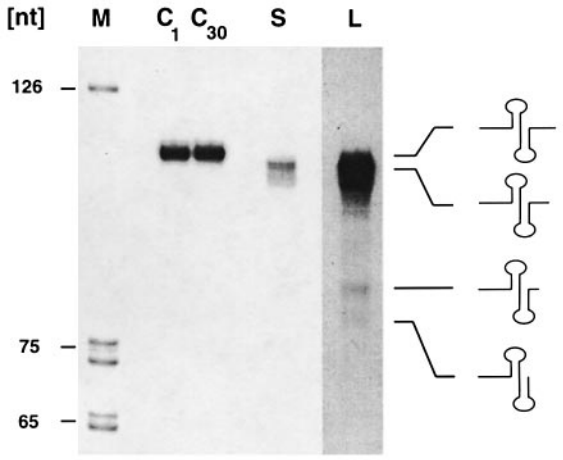

Fig. 2. The IR sequence destabilizes adjacent downstream transcript sequences in the *in vitro* transcription lysate. To test whether the RNA molecules smaller than the run-off transcripts observed in the *in vitro* transcription (Figure 1) are indeed generated by specific degradation, 96 nucleotide long RNAs synthesized from T7 promoters were incubated with the *in vitro* transcription lysate (lane S is a short, lane L a long exposure). The RNA molecule distribution generated is similar to that observed in the *in vitro* transcription (Figure 1), suggesting that the shorter molecules are indeed derived exclusively by post-transcriptional processing. Long exposure (lane L) reveals a very small amount of RNAs with 3′-ends close to the IR structure. The schematic drawings on the right identify the different RNA molecules of assayed RNA, intermediates and processed RNA. Control incubation (C) without lysate for 1 or 30 min respectively shows no degradation of the RNA.

through the respective sense constructs. The relative amounts of the intermediate products and of the apparently correctly trimmed molecules terminating just close to the IR appear to be distributed similarly to those in the assays with the sense IR templates. Thus the spatial structure rather than the primary RNA sequence determines recognition of the IR element as a processing signal in plant mitochondria.

A complex mitochondrial lysate efficiently matures 3′-termini

To investigate the potential for more efficient reaction conditions, *in vitro* processing of RNA substrates containing the pea *atp9* IR was tested in more complex mitochondrial lysate fractions. Incubation with such preparations indeed yielded the RNA degradation products much more efficiently (Figure 4). Correct products of ~68 nucleotides, coinciding in length with a product shortened downstream of the IR, rapidly accumulate and represent the most prominent RNA species generated during the *in vitro* processing reaction.

The time course of the reaction reveals far fewer intermediate products appearing transiently than in the *in vitro* transcription fraction. Upon prolonged incubation, further degradation of the RNA with some slightly preferred degradation intermediates becomes apparent, as expected in such complex organellar protein mixtures (data not shown). However, none of the observed RNA degradation products match the 28 nucleotide long RNA expected for the 3′-trailer upon endonucleolytic cleavage, suggesting either an extremely rapid breakdown of this trailer to mononucleotides or exonucleolytic processing from the 3′-end of the precursor RNA.

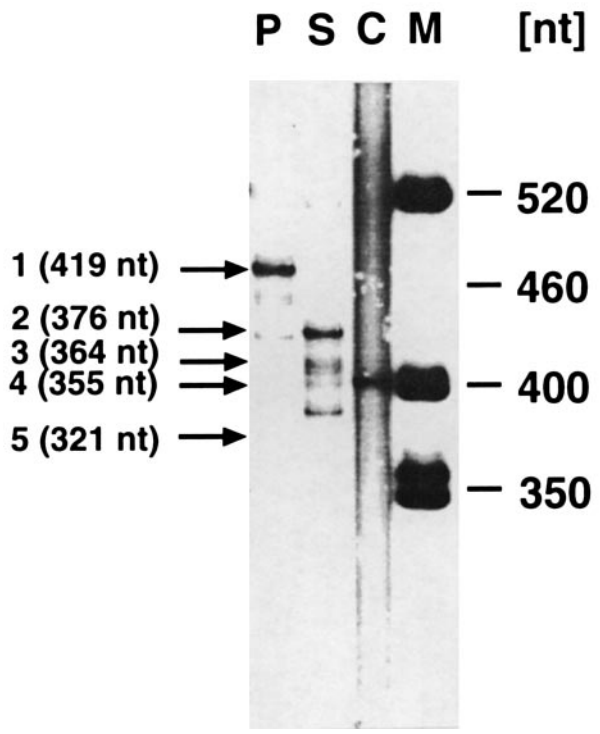

Fig. 3. The spatial structure and not the primary sequence of the IR is essential for its function. To differentiate between the influence of the structure of the IR and the primary sequence of this region, constructs containing the IRs in antisense orientation were incubated with a mitochondrial *in vitro* transcription lysate. In the pea (lane P) as well as in the soybean (S) context, the antisense IR does not terminate transcription efficiently. However, it induces the generation of smaller RNA molecules by successive 3′ degradation, while the control RNA (C) without the repeat is comparatively stable. *In vitro* transcription template constructs are as in Figure 1A and C, but with the *Cla*I fragment in the opposite orientation. Transcript 1, run-off from the pea template; transcript 2, run-off from the soybean template; transcript 3, expected RNA length at the end of the IR (pea); transcript 4, control RNA without IR; transcript 5, expected RNA length at the end of the IR (soybean).

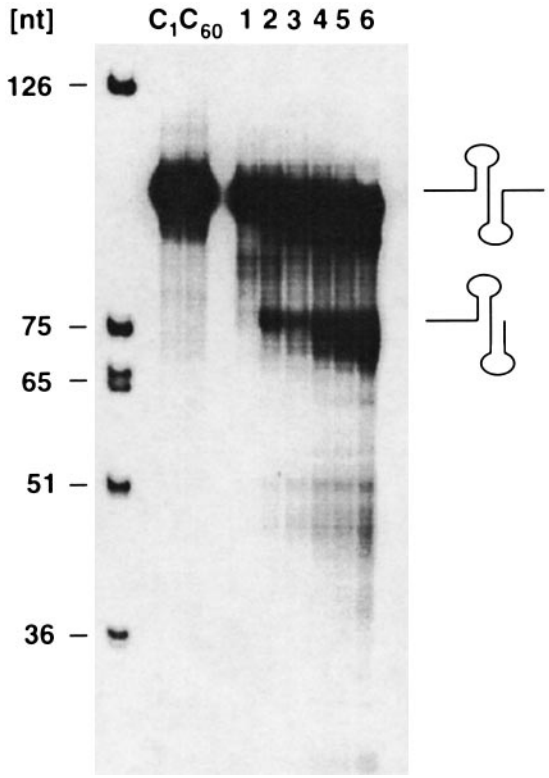

Fig. 4. RNA molecules are processed efficiently in the pea mitochondrial *in vitro* processing extract. With increasing incubation periods (lanes 1–6: 1, 5, 10, 20, 30 and 60 min) RNA molecules synthesized from T7 promoters are increasingly degraded to products ending just downstream of the IR structure. This mitochondrial lysate thus contains all specificity determinant(s) for efficient and correct IR recognition and processing. Prolonged incubation degrades the RNA further to apparently unspecific intermediate products. C_1 and C_{60} are control incubations of the RNA without lysate for 1 and 60 min respectively. Structures of the incubated RNA and the resulting product are depicted on the right. A DNA standard is shown on the left.

The in vitro-generated 3′-termini match in vivo mRNA ends

Although the length estimations of the processing products generated *in vitro* suggest processing near the IR structure, the resulting 3′-termini still needed to be determined more precisely and had to be compared with the *in vivo* termini. The 3′-ends of *atp9* transcripts were therefore investigated by S1 protection in a direct comparison of the *in vivo*- and *in vitro*-generated RNAs (Figure 5). This experiment shows the distribution of the 3′-RNA termini to be identical between the *in vitro*- and *in vivo*-generated RNAs. In both systems, the terminal nucleotides are scattered over a few nucleotides at the 3′-side of the IR region, showing identical distributions of major and minor RNA termini.

Altering the ratio between RNA and nuclease S1 by increasing the quantity of the *in vivo* mitochondrial RNA (Figure 5, lane 1) reveals the presence of larger RNA molecules in mitochondria. This observation supports the conclusion that the IR structures can also be passed by the transcription process *in vivo*.

Discussion

The IR structures downstream of coding regions in plant mitochondrial genes do not terminate transcription

The hitherto unclear function of the IR structures was analyzed by means of *in vitro* transcription and *in vitro* processing assays. Investigation of the transcriptional processivity *in vitro* shows that, at least *in vitro*, transcription is not terminated effectively by the IR structure, but that the majority of the transcripts continue unimpeded into the sequence beyond (Figure 1). The derivation of the smaller RNA molecules from secondary degradation of full-length run-off RNAs is confirmed by incubation of pre-synthesized transcripts in this same lysate (Figure 2). The presence of RNA molecules *in vivo* extending beyond the IR structure (Figure 5) confirms that this structure does not represent an absolute steric barrier for transcription also *in vivo*.

The very similar patterns of intermediates and mature processing products in the *de novo* transcription and of added RNA molecules in the *in vitro* transcription lysates suggest that termination of transcription does not con-

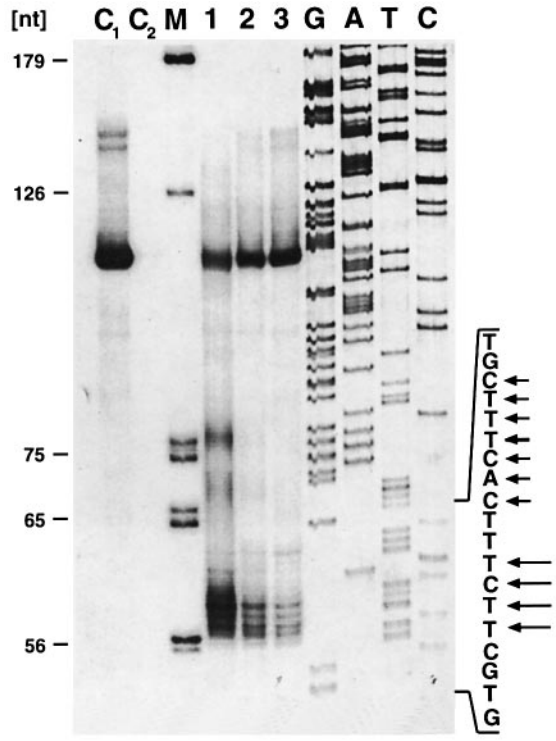

Fig. 5. Comparison between *in vivo*- and *in vitro*-generated 3′-RNA termini at the pea mitochondrial IR structures. The precise RNA 3′-termini were compared at the nucleotide level between the *in vivo* pea *atp9* mRNA (lane 2 with 20 μg and with five times the quantity of mitochondrial RNA, 100 μg, in lane 1) and the *in vitro* product generated in the processing lysate (lane 3). The protected DNA molecules show virtually identical patterns for *in vivo* and *in vitro* RNAs. The identical *in vivo* and *in vitro* termini (long arrows) locate in the far stem. The larger RNA molecules seen in lane 1 with an increased amount of RNA show that longer RNA molecules do exist *in vivo*, confirming that *in vivo* transcription can also proceed through the IR. Short arrows indicate the very close positions of the *atp9 in vivo* termini mapped independently and with different S1 conditions (Morikami and Nakamura, 1993). Controls are, in lane C_1, the DNA fragment without S1 and RNA and, in C_2, the DNA fragment digested with S1 but without RNA. Sizes of a DNA marker (M) are given in nucleotides on the left. The sequence ladder (G,A,T,C) was generated from the same primer atp9-1 as the S1 templates.

tribute detectably to the observed reaction products. A role for these IRs as transcription terminators in plant mitochondria is therefore highly unlikely, although it is still possible that a specific transcription termination factor could be missing from the mitochondrial lysates.

The bacterial recognition of such structures as transcriptional terminators (Manley and Proudfoot, 1994; Cohen, 1995) appears to have been lost in plant mitochondria as well as in chloroplasts. In the latter, such stem–loop structures have also been found to act as mRNA processing signals rather than terminating transcription. However, in these *in vitro* tests, a residual influence on transcriptional pausing and release of the nascent RNA may be effected by these IR tracts since, in the *Chlamydomonas* chloroplast, *rbcL* and *psaB* IRs appear to terminate transcription *in vivo* (Blowers *et al.*, 1993) and bacterial terminators such as the *thra* structure are still a hindrance to a spinach chloroplast RNA polymerase (Chen *et al.*, 1995).

In plant mitochondria, the precise transcription termination found in bacteria appears to have been superseded by the tightly regulated control of processing. Thus, transcription may continue for quite a long distance along the mitochondrial genome up to as yet unclear steric obstacles, with processing defining mature and stable transcripts. Processing most likely also uses additional signals, since not all 3′-transcript termini locate at stem–loop-forming IR structures.

IRs induce degradation of downstream RNA regions

Transcripts synthesized in the pea *in vitro* transcription system (Binder *et al.* 1995) appear to be rather stable and show little degradation (Figure 1B and D, control lanes C). Introduction of an IR structure, however, partly destabilizes transcripts and results in several smaller RNA species. The smallest of these RNAs still contains the IR element and has been shortened by the entire 3′-extension. The co-linearity between probe and product is confirmed by the S1 protection experiments (Figure 5). The IR thus appears to destabilize the 3′-region downstream of the stem–loop and to induce its successive degradation, as evidenced by the appearance of intermediate RNA species. This selective destabilization is confirmed by incubation of RNA molecules in the *in vitro* processing lysate, which rapidly dissociates the 3′-terminal nucleotides up to the IR structure, but not the stem–loop itself and upstream sequences.

The presence of the IR structure seems to stabilize upstream sequences, since RNA molecules without such an IR are degraded without any discernible pausing or detectable intermediate species (Figure 1B and D, control lanes C). In plant mitochondria, these IRs appear to have a dual function, assembling the RNase/processing activity(s) and at the same time protecting the upstream RNA moiety, regulating controlled processing of mRNAs in these organelles.

Requirements for IR recognition and an efficient mitochondrial in vitro processing lysate from pea

Exchange of the sequences upstream of the IR shows that indeed the IR element rather than the upstream sequence environment carries the essential information. Even the interspecies exchange between pea and soybean contexts leaves its function undisturbed. Incubation of the antisense IR sequence, furthermore, shows the destabilizing/stabilizing function of the IR to reside in the structure rather than in a particular primary sequence (Figure 3). However, additional sequence-specific recognition elements similar to 3′-IR recognition in plastid RNAs may play modulating roles and distinguish individual (or classes of) transcripts through sequence-preferred binding properties (Stern and Gruissem, 1987; Rochaix, 1996; Sugita and Sugiura, 1996).

Incubation of pre-synthesized RNA molecules in the *in vitro* transcription lysate preparation from pea mitochondria does yield the correct mRNA terminus, but in rather low abundance and in fairly low quantities. The conclusion that one or more specificity/activity factor(s) are depleted in this lysate is confirmed by more efficient processing of the precursor molecules in the less specifically sorted mitochondrial lysate prepared for *in vitro* processing.

This RNA-processing lysate from pea mitochondria competently matures added precursor RNA molecules to

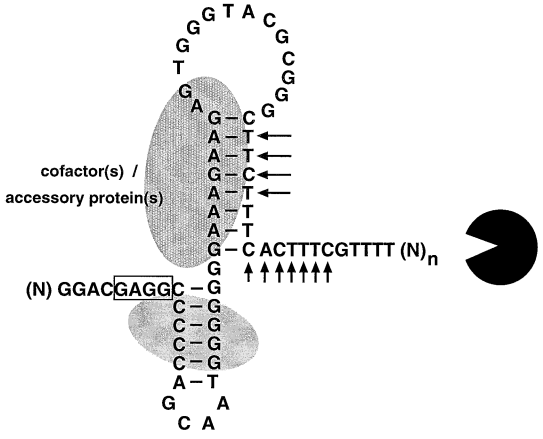

Fig. 6. Secondary structure model of the pea *atp9* 3′-IR as a processing signal in plant mitochondria. Transcript 3′-termini generated by *in vitro* processing coincide with the *in vivo* RNA termini (long arrows) and are located in the furthest stem of the double stem–loop structure predicted for this IR. Short arrows indicate longer transcripts identified *in vivo* (Morikami and Nakamura, 1993). Accessory protein(s) and/or co-factor(s) at the same time protect the IR and upstream sequences and guide RNase(s) (open mouth) to the 3′-region. These are most likely exonucleases, as interpreted from the apparent 3′ to 5′ processivity. A 5′-GAGG-3′ motif (boxed) is found in several plant mitochondrial genes at this position just preceding the first stem of the respective IR.

the 3′-side of the IR structure, yielding termini identical to the *in vivo* steady-state transcripts (Figures 4–6). The variation in the *in vivo* termini determined in this and a previous analysis (Morikami and Nakamura, 1993) is most likely due to the different S1 conditions used. The lysate appears to contain guiding co-factor(s) as well as the attracted RNase(s) and, at the same time, factor(s) essential for stabilizing the IR and the upstream RNA sequence.

Exonucleolytic removal of downstream nucleotides

The consistently observed presence of intermediate molecule sizes between the run-off transcripts and the smaller matured RNAs terminating just downstream of the IR suggests successive removal of the 3′-nucleotides rather than an endonucleotlytic cut just at the IR (Figures 1 and 2). Furthermore, no RNA molecule of the size expected for the 3′-trailer is observed, in any of the processing experiments, above the unspecific degradation background, even upon very long exposure (Figure 2, lane L; and data not shown). Such endonucleolytically derived RNA products are stable enough in plant mitochondrial *in vitro* lysates to be readily detectable, as seen in tRNA processing assays of wheat mitochondrial lysates (Hanic-Joyce and Gray, 1990). Unless this trailer region is selectively hydrolyzed more rapidly, exonucleolytic digestion rather than an endonucleolytic cut is the most likely explanation. Another argument for exonucleolytic removal of the 3′-region is the presence of the scattered nucleotides observed in the S1 protection experiments (Figure 5). However, such a scattering of RNA termini could, alternatively, be attributed to S1 artifacts, frequently generated by this enzyme (Figure 5). Slowly acting additional endonucleases may contribute to processing at other 3′-termini, but may be masked by the rapid exonucleases at this particular IR sequence.

Structure rather than nucleotide sequence appears to guide RNA degradation/maturation in plant mitochondria

The 3′-located stem–loop structures in plant mitochondrial RNAs are engaged as processing signals during maturation of mRNAs. The accompanying manuscript extends the observations made here for pea mitochondria to another dicot, demonstrating the processing/stabilizing effect of a different IR in *Brassica* cybrids. Taken together, these results show RNA stability to contribute to the regulation of mitochondrial gene expression in plants.

The studies presented here for pea and in the accompanying work for *Brassica* extend the indirect observations of differential RNA stability being conferred by genomic differences between the 3′-mRNA structures of *cob* genes *in vivo* in rice (Kaleikau *et al.*, 1992) and wheat lines (Saalaoui *et al.*, 1990).

At present, we cannot decide how much the accelerated degradation of the RNA region 3′ to the IR and the enhanced stability of the RNA sequence upstream of the IR respectively contribute to regulating the quantity of mature transcripts in plant mitochondria. Primary sequence features, although not apparent in the antisense IR transcript, may well be of *in vivo* importance, since the *atp9* 3′-IR region itself is well conserved between pea and broad bean in both primary sequence and secondary structure (Wahleithner and Wolstenholme, 1988). In particular, the 5′-GAGG-3′ element just 5′ to the IR region (Figure 6) could be an important element, since it is found in several IRs from very different plants (Schuster *et al.*, 1986; Wahleithner and Wolstenholme, 1988). The precise sequence features need to be dissected in detail, as has been done for plastid RNA maturation in algae and higher plants (Stern and Gruissem, 1987; Rochaix, 1996; Sugita and Sugiura, 1996).

Are 3′-IR processing features similar in plant mitochondria, plastids and bacteria?

The RNA synthesizing and processing pathways of the bacterial endosymbiont were introduced into the eukaryotic host cell with the integration of the endosymbiont. In the plant cell, two such systems were imported independently with the plastid and the mitochondrion, respectively (Herrmann, 1996). Several distinct scenarios are possible regarding how RNA abundance is regulated in plant cell organelles: firstly, regulatory factors may be the original ones of the respective endosymbiont; secondly, some components may have double functions and be used in both compartments; and, thirdly, host factors of the eukaryotic nucleus may have been recruited for the organelle(s). Thus, in investigating transcript processing and stability control in plant mitochondria, we must be aware of individual components being of either mitochondrial, chloroplast or eukaryotic origin.

In the comparison of the three genetic systems, plastids appear to have retained more bacterial features than the mitochondria, particularly when considering transcription initiation and the translational apparatus. The proteins identified in the mRNA processing/stabilizing complex in plastids are likewise mostly clearly recognizable homologs of bacterial factors (Hayes *et al.*, 1996). It will thus also be of evolutionary interest to identify and compare the

individual proteins involved in the post-transcriptional control of mRNA maturation in plant mitochondria.

Materials and methods

Template construction

The double inverted repeat located in the 3′-untranslated region of the mitochondrial pea *atp9* gene (Morikami and Nakamura, 1993) was amplified by PCR between primers PA-11 (5′-GTATCGATGTAGTCCC-TGAGGAC-3′) and PA-12 (5′-TTATCGATGCTCTCTCCAAAACG-3′) from a clone containing the type I allele of the *atp1*/*atp9* arrangement as template (Morikami and Nakamura, 1987). The resulting DNA fragment (102 bp) was digested with *Cla*I and cloned in both orientations into the respective sites in clones patp9SC550 and satp9XR482 (Binder *et al.*, 1995). In clone patp9SC550, the fragment was inserted into the *Cla*I site 294 bp downstream of the pea *atp9* promoter. In clone satp9XR482, the IR was cloned into a *Cla*I site located in vector sequences 251 bp downstream of the soybean *atp9* promoter. Correct insertion and orientation of the IRs was monitored by DNA sequencing. Cloning procedures followed standard protocols (Sambrook *et al.*, 1989).

In vitro transcription assay

Transcriptionally active mitochondrial lysates and *in vitro* transcription assays with mtDNA templates were prepared and assayed as described previously (Binder *et al.*, 1995).

Preparation of pea mitochondrial processing extracts

For *in vitro* processing assays, mitochondrial protein lysates were prepared from mitochondria isolated from 6- to 7-day-old etiolated pea seedlings (*Pisum sativum*, var. Lancet) by differential centrifugation and purification on Percoll gradients. About 2 g of mitochondria were lysed in the presence of 0.5% Triton X-100 and 1 M KCl. Insoluble components were pelleted in a centrifugation step at 100 000 *g* and the supernatant was diluted with 1 vol. of buffer V containing 90 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT) and 2.5% glycerol. Hydrophobic proteins were removed from this suspension by adding solid $(NH_4)_2SO_4$ to a final concentration of 20% (w/v) and centrifugation at 16 000 *g* for 30 min. Proteins were precipitated from the supernatant by adding solid $(NH_4)_2SO_4$ to saturation and centrifugation at 16 000 *g* for 90 min. Pelleted protein was resuspended in 1 ml of buffer A (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 7.5% glycerol and 50 mM KCl) and dialyzed against 2 l of the same buffer. Aliquots of this fraction were frozen rapidly in liquid nitrogen and stored at –80°C. Protein concentrations were determined with the Bradford protein assay (Bio-Rad).

RNA substrate preparation

The DNA template for *in vitro* synthesis of mRNA containing the pea *atp9* double stem–loop with 39 nucleotides upstream and 28 nucleotides downstream was obtained by PCR with primers T7IVR+ (5′-TAAT-ACGACTCACTATAGGTGTAGTCCCTGAGGAC-3′) and PA-12. The resulting 114 bp fragment contains the T7 promoter sequences of 18 nucleotides integrated into primer T7IVR+. The reaction parameters were 40 cycles of 1 min at 94°C, 1 min at 42°C and 1 min at 72°C followed by a final elongation at 72°C for 5 min. PCR products were extracted with phenol/chloroform and precipitated by ethanol.

About 2 µg of template DNA were used in the *in vitro* transcription reaction with T7 RNA polymerase in the presence of [α-^{32}P]UTP (3000 Ci/mmol). Reactions were carried out using an RNA transcription kit according to the manufacturer's protocol (Stratagene). Transcription products were purified by electrophoresis on 5% denaturing polyacrylamide gels followed by elution overnight at room temperature and precipitation by ethanol. The *in vitro*-generated mRNA was finally resuspended in double-distilled H_2O, and incorporation of labeled UTP was measured by scintillation counting.

In vitro processing reactions

In vitro processing assays were carried out in a total reaction volume of 15 µl. The reactions were performed with 75 µg of mitochondrial protein and 15 000 c.p.m. of internally labeled *in vitro* T7-generated pre-mRNA in the presence of 10 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and 10 µg/ml of heterologous tRNAs prepared from wheat germ. After incubation at 30°C for up to 60 min, reactions were stopped by adding 45 µl of stop solution (4.8 M urea, 0.4 M sodium acetate, 5.3 mM aurintricarboxylic acid, 26 µg/ml heterologous tRNAs), extracted with phenol/chloroform, and nucleic acids were precipitated with ethanol. After resuspension in 5 µl of loading solution, the processing products were co-electrophoresed with a labeled double-stranded DNA length standard on 7% denaturing polyacrylamide gels and detected by autoradiography.

S1 nuclease protection analysis

The DNA fragment used in the S1 protection analysis of the 3′-ends of *in vitro* processing products and of mitochondrial *in vivo atp9* RNAs was generated by PCR with primers atp9-1 (5′-GAGGACGAGGC-CCCCAGC-3′) and Reverse (5′-GGAAACAGCTATGACCATG-3′) and 35 cycles with the following parameters: 1 min 94°C, 1 min 41°C and 1 min 72°C. The 3′-ends of the DNA fragment were labeled with [α-^{32}P]ddATP and terminal transferase under conditions recommended by the manufacturer (Boehringer). *Kpn*I-digested DNA fragments were separated on a 2% agarose gel, eluted from the gel, extracted with phenol/chloroform and ethanol precipitated.

Total mtRNA was isolated from mitochondria as described (Binder and Brennicke, 1993). *In vitro* processing products were eluted from a polyacrylamide gel slice in a buffer containing 0.5 M sodium chloride, 0.1 mM EDTA, 0.1% SDS and 1% phenol and were precipitated with ethanol. *In vivo* mtRNA and *in vitro* processed RNA, respectively, were hybridized to the 3′-labeled antisense DNA at 20°C for 14 h, followed by digestion with 150 U of S1 nuclease at 37°C for 30 min. Reactions were terminated by phenol/chloroform extraction and ethanol precipitation. Protected DNA fragments were resuspended in loading solution and co-electrophoresed with a DNA length marker, and DNA sequencing products were obtained with primer atp9-1 on an 8% polyacrylamide gel.

Miscellaneous techniques

Plasmid DNA was prepared and purified on CsCl gradients as described elsewhere (Sambrook *et al.*, 1989). Restriction digests were performed according to the manufacturer's recommendations. Plasmid DNA was sequenced with a Thermo Sequenase fluorescent label primer cycle sequencing kit according to the the manufacturer's protocol (Amersham). Fluorescently labeled sequencing products were analyzed on an ALFexpress sequencer (Pharmacia). Radioactive sequencing reactions were done with a T7 sequencing kit (Pharmacia).

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