The steady-state level of mRNA from the Ogura cytoplasmic male sterility locus in *Brassica* cybrids is determined post-transcriptionally by its 3' region

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We have investigated the control of the expression of three different configurations of the mitochondrial gene *orf138*, whose expression is correlated with Ogura cytoplasmic male-sterility in rapeseed cybrids. These configurations, termed *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S*, specific to the 13S (sterile), 13F (fertile) and 18S (sterile) cybrids respectively, have the same 5' regions but different 3' regions. The *orf138* transcript from *Bam4.8/18S* is 10-fold more abundant than the one from *Nco2.5/13S*, while no *orf138* transcript from *Nco2.7/13F* accumulates. However, transcriptional activity measurements show that the rate of transcription is equivalent for the three configurations. These results strongly suggest that the steady-state level of mRNA from the *orf138* locus is determined post-transcriptionally, most likely by its 3' region. To determine the role of these 3' regions, we have established an *in vitro* decay and processing system. In the presence of rapeseed mitochondrial lysate, synthetic RNAs corresponding to the 3' region of the *Nco2.7/13F* transcript are, as expected, less stable than RNAs corresponding to the 3' regions of the *Nco2.5/13S* and *Bam4.8/18S* transcripts. We have also observed *in vitro* processing of synthetic RNAs at the sites corresponding to the 3' ends of the natural mRNAs from *Nco2.5/13S* and *Bam4.8/18S*. Further analysis of the role of these 3' regions in *in vitro* RNA stability should help us to better understand post-transcriptional control in plant mitochondria.

Keywords: cybrids/cytoplasmic male-sterility/ mitochondrial gene expression/mRNA stability/3' end processing

Introduction

In mammals and fungi, the control of the expression of the mitochondrial genome has been extensively investigated, while relatively little is known about the control of the expression of plant mitochondrial genes. Recently, however, important progress has been achieved in the identification of plant mitochondrial promoter sequences (Binder *et al.*, 1990; Brown *et al.*, 1991; Covello and Gray, 1991; Mulligan *et al.*, 1991; Binder and Brennicke, 1993; Nakazono *et al.*, 1995). *In vitro* transcription systems have been established and allow the identification of

sequences necessary and sufficient for efficient transcription initiation (Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992; Rapp *et al.*, 1993; Binder *et al.*, 1995). Nevertheless, previous work has demonstrated that, as in plant chloroplasts, post-transcriptional control plays an important role in the regulation of plant mitochondrial genome expression (Finnegan and Brown, 1990; Mulligan *et al.*, 1991). Almost nothing is known about the mechanisms that control gene expression at this level. Like chloroplast transcripts, plant mitochondrial mRNAs tend to contain inverted repeat sequences in their 3' region that can fold into stem-loop structures (Bland *et al.*, 1986; Schuster *et al.*, 1986; Rothenberg and Hanson, 1987; Wahleithner and Wolstenholme, 1988; Wissinger *et al.*, 1988; Gualberto *et al.*, 1990; Macfarlane *et al.*, 1990; Saalaoui *et al.*, 1990; Kaleikau *et al.*, 1992; Liu *et al.*, 1992). In *Chlamydomonas* and higher plant chloroplasts, these inverted repeats act to stabilize upstream sequences (Stern and Gruissem, 1987; Stern *et al.*, 1989, 1991; Adams and Stern, 1990; Stern and Kindle, 1993) and interact with enzyme complexes required for 3' end processing (Hayes *et al.*, 1996). In plant mitochondria, the role of the 3' untranslated region of transcripts is still unknown.

Protoplast fusion is a very powerful tool for the manipulation of plant mitochondrial genomes (Belliard *et al.*, 1979), allowing genetic recombination between mitochondrial genomes of different species and leading to new associations of sequences. In this paper, we describe studies which were conducted on *Brassica napus* cybrids obtained after fusion of protoplasts from plants bearing normal *Brassica* cytoplasm and plants bearing Ogura radish cytoplasmic male sterility (CMS)-inducing cytoplasm (Bannerot *et al.*, 1977; Pelletier *et al.*, 1983). CMS is a maternally inherited, mitochondrially encoded trait that prevents the production of functional pollen but maintains female fertility (Vedel *et al.*, 1994). The comparison between the closely related mitochondrial genomes of male-sterile (13S) and fertile revertant (13F) progenies of a cybrid allowed the identification of a sequence (a 2.5 kb *NcoI* fragment termed *Nco2.5/13S*) associated with Ogura CMS (Bonhomme *et al.*, 1991). Two open reading frames (*orf138* and *orfB*) were found in this fragment, and *orf138* expression was correlated with Ogura CMS in rapeseed cybrids (Bonhomme *et al.*, 1992). Grelon *et al.* (1994) demonstrated that *orf138* is translated into a mitochondrial membrane protein in sterile cybrids. The fertile revertant cybrid 13F contains the *orf138* gene on the *Nco2.7/13F* fragment; no transcripts from this gene accumulate and the *orf138* protein is not detectable (Bonhomme *et al.*, 1992; Grelon *et al.* 1994). A third configuration of the *orf138* gene (*Bam4.8/18S*) was found in another sterile cybrid, termed 18S (Grelon *et al.*, 1994). Previous Northern blot analyses have shown differences in the steady-state

Fig. 1. Restriction maps of the three configurations *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S*, specific to the 13S (sterile), 13F (fertile) and 18S (sterile) cybrids respectively. The sequences of the *Nco2.7/13F* and *Bam4.8/18S* fragments which are common with the *Nco2.5/13S* fragment are indicated by a thick black line. White box: *trnfM* gene coding for initiator methionine tRNA; light grey box: *orf138* gene; dark grey box: *orfB* gene; striped box: *atpA* gene. *In vivo* accumulated transcripts from each configuration are indicated by the bent arrows.

orf138 mRNA accumulation between the three configurations *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S* specific to the 13S, 13F and 18S cybrids respectively (Grelon *et al.*, 1994). The three configurations of the mitochondrial gene *orf138* have been shown to have the same 5' region but different 3' regions (Figure 1). The differences between these three configurations make these cybrids ideal material for examining the role of 3' untranslated regions in plant mitochondrial genome expression.

In this report we show that the expression of the genes at the CMS locus is determined post-transcriptionally. Our data suggest that the 3' region of the *orf138* transcripts plays an important role in transcript processing and stability.

Results

Expression of the *orf138* gene in *Brassica* cybrids

Steady-state level of *orf138* mRNA. To quantify the differences in the steady-state *orf138* mRNA accumulation between the three configurations *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S*, slot blots were carried out with controlled quantities of total RNA from the three cybrids. Hybridization with an *orf138* probe showed that the *orf138* transcript from *Bam4.8/18S* in sterile cybrid 18S is 10-fold more accumulated than the one from *Nco2.5/13S* in sterile cybrid 13S, whilst no *orf138* transcript is detected from *Nco2.7/13F* in cybrid 13F (Figure 2A). To determine whether these differences in gene expression are due to differences in mitochondrial DNA copy number, *orf138* abundance in total DNA was quantified on a slot blot carrying total DNA. Figure 2B shows that the ratio

Fig. 2. Differential abundance of the *orf138* mRNA is not accompanied by differences in mitochondrial DNA copy number in *Brassica* cybrids. Slot blots of total RNA (A) or total DNA (B) were hybridized successively with radiolabelled *orf138* and *atpA* probes (*atpA* was used as an internal control). Each lane contains decreasing quantities (10 µg, 1 µg, 100 ng) of total RNA or total DNA of 13S, 13F or 18S cybrids.

between *orf138* and *atpA* signals is approximately identical for the three cybrids. The *orf138* locus is, therefore, present in an equal number of copies in the three cybrids. Thus differential amplification of the *orf138* locus in the three cybrids cannot account for the differences in steady-state accumulation of *orf138* mRNA.

Fig. 3. An *orf138-atpA* co-transcript is synthesized from the *Nco2.7/13F* configuration. RT-PCR experiment carried out using the two primers A and B, shown in the lower panel of the figure on the restriction map of *Nco2.7/13F*. The substrate was total RNA from buds of the 13F cybrid following reverse transcription (+RT) or without reverse transcription (-RT). The size of the PCR amplified fragment with the reverse transcribed sample is indicated. Lane M contains marker fragments (1 kb ladder BRL).

A transcript is synthesized from *Nco2.7/13F* but it does not accumulate. To check for the presence of an *orf138* transcript from *Nco2.7/13F*, RT-PCR experiments were performed on RNA from the 13F cybrid (Figure 3). An amplified fragment of the expected size (1100 bp) was obtained when cDNA from the 13F cybrid was used as template (+RT). This fragment was not obtained when the 13F RNA sample was not previously incubated with reverse transcriptase (-RT). Cloning and sequencing of the PCR product revealed that the 1100 bp fragment contains the *Nco2.7/13F* sequence and thus is derived from an *orf138* transcript. This sensitive experiment shows that an *orf138* transcript is synthesized from *Nco2.7/13F*, although it is not detectable on Northern or slot blots, and that *orf138* is co-transcribed with *atpA* in this configuration.

Nco2.5/13S, *Nco2.7/13F* and *Bam4.8/18S* are transcribed at the same rate. Since the three configurations *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S* have the same 5' region, it is likely that these configurations have identical promoters and thus could be transcribed at equal rates. To test this hypothesis, run-on experiments were used to compare the transcription of *orf138* in the three configurations (Figure 4). Isolated mitochondria from 13S, 13F and 18S cybrids were incubated with radiolabelled nucleotides. The run-on products, which reflect the elongation of pre-existing transcriptional complexes (Mulligan *et al.*, 1991), were hybridized to slot blots carrying the *orf138* and *atp9* gene sequences. Quantitation analysis by phosphorimaging

Fig. 4. The *orf138* gene is transcribed at the same rate in the three configurations. Run-on transcription products synthesized by mitochondria from 13S, 13F or 18S were hybridized to slot blots carrying 4 µg of *orf138*, *atp9* (used as an internal control) and pBluescript SK⁻ plasmid (used as negative control). After hybridization, the membranes were treated with RNase, washed and then autoradiographed.

indicated that the ratio between *orf138* and *atp9* signals is approximately identical for the three cybrids. The *orf138* gene has, therefore, a similar rate of transcription in the three cytotypes. Thus, differential gene expression must reflect post-transcriptional events.

Analysis of the 5' and 3' termini of the *orf138* transcripts

Determination of the 5' end of the *orf138* transcripts. Primer extension experiments were used to map the 5' ends of the transcripts from *Bam4.8/18S* and *Nco2.5/13S* (Figure 5A). Using total RNA from 18S and 13S as templates, three primer extension products were obtained. This experiment showed that the *orf138* transcripts from *Bam4.8/18S* (in 18S mitochondrial RNA) and *Nco2.5/13S* (in 13S mitochondrial RNA) have identical 5' termini. A sequence reaction using a subclone of the *orf138* locus and the primer used in primer extension allowed the precise location of the 5' termini at the C, G and U nucleotides 107, 106 and 105 nucleotides respectively from the initiation AUG of the *orf138* coding region. Although we did not obtain primer extension products for the 13F cybrid, we assume that the *orf138* transcripts from *Nco2.7/13F* have the same 5' termini. These 5' termini are 3, 4 and 5 bp upstream of the 5' end previously suggested for the *Nco2.5/13S* transcript (Bonhomme *et al.*, 1992). Since we used a sequence ladder to size our primer extension products, we suggest that our mapping is more accurate.

To determine if the 5' ends of the *orf138* transcripts are processing sites or transcription initiation sites, *in vitro*-capped mitochondrial RNA and 5'-phosphorylated mitochondrial RNA were hybridized to restriction fragments of the cloned 5' region of the *orf138* locus. The capping enzyme guanylyltransferase specifically labels the 5' di- or triphosphate termini of primary transcripts (Christianson and Rabinowitz, 1983), while the polynucleotide kinase enzyme labels the monophosphate 5' ends formed by RNA processing. The *HincII-XbaI* fragment (325 bp) containing the 5' ends of the *orf138* transcripts was labelled after hybridization with phosphorylated mitochondrial RNA, but was not labelled after hybridization with capped RNA (Figure 5B). The capped RNA used in this experiment was also hybridized to an *orfB* fragment and gave a similar result to the experiment shown in Figure 7C (data not shown), showing that the capping of the

Fig. 5. The 5' termini of the *orf138* transcripts are identical in cybrids 13S and 18S, and are processing sites. **(A)** 5' end mapping of the *orf138* transcripts. Labelled oligonucleotide PE, indicated below the figure on the restriction map of the 5' region of the *orf138* locus, was hybridized to total RNA of 13S, 13F and 18S. After reverse transcription, the primer extension products were separated on a 6% sequencing gel next to sequencing reactions using PE as primer and the 5' region of the *orf138* locus (subcloned in Bluescript) as template. The sequence surrounding the 5' ends is given on the right. The arrows correspond to the 5' ends and the boxed sequence corresponds to the end of the *trnfM* sequence. **(B)** Hybridization of phosphorylated or capped mitochondrial RNA from the 18S cybrid. After hybridization of labelled mitochondrial RNA to a Southern blot of the *HincII*–*XbaI* subclone, the blots were treated with RNase, washed and autoradiographed. The labelled mitochondrial RNA used in each case is indicated above each panel.

mitochondrial RNA was successful. Thus, the 5' ends of the *orf138* transcripts are produced by a processing event, and transcription of the *orf138* locus probably initiates upstream of the adjacent *trnfM* gene. This result was expected because the flanking sequences of the 5' end of

orf138 transcript show no similarity to the dicot consensus sequence for transcription initiation (Brown *et al.*, 1991; Binder and Brennicke, 1993).

A tRNA-like element is present immediately downstream of the 3' ends of the *orf138* transcript from *Bam4.8/18S*. In order to identify the 3' end of the *orf138* transcript in *Bam4.8/18S*, an antisense probe was used in RNase mapping experiments (Figure 6A). Two ladders of several bands were obtained from 18S mitochondrial RNAs. For each ladder, two major protected fragments were seen (91–94 and 73–74 nucleotides). After a long exposure of the gel, another protected fragment (164 nucleotides) was seen which may represent a minor 3' end. Using the MFOLD software in the GCG package, a tRNA-like structure can be formed by folding the 3' untranslated region of the *Bam4.8/18S* (Figure 6B). Similar tRNA-like structures have been identified in wheat mitochondrial DNA and have been termed 't-elements' (Hanic-Joyce *et al.*, 1990). Interestingly, the t-element sequence is identical in size to the smallest protected product obtained in the RNase protection experiments (Figure 6A). Thus, the 73–74 protected fragments probably reflect RNase protection of the processed t-element rather than protection by the 3' untranslated region of the *orf138* transcripts from *Bam4.8/18S*. The minor protection fragment is the right size to represent an *orf138* transcript including the t-element; the major 3' end corresponds to *orf138* transcripts lacking the t-element. This interpretation is confirmed by Northern experiments. A probe complementary to the t-element does not hybridize to *orf138* transcripts, but does recognize a tRNA-sized transcript in mitochondrial RNA of plants carrying the *Bam4.8/18S* fragment (Figure 6C).

A processing event between the *orf138* and *atpA* coding regions is responsible for the formation of the 3' end of the *orf138* transcript from *Nco2.7/13F*. RT-PCR experiments have shown that a co-transcript *orf138-atpA* is synthesized from *Nco2.7/13F* (Figure 3), and primer extension experiments revealed that the 5' end of the *atpA* transcript is localized 199 nucleotides upstream of the initiation AUG of the *atpA* coding region (data not shown). To check the hypothesis that the monocistronic *atpA* transcript is formed by RNA processing of the *orf138-atpA* co-transcript, the nature of the 5' end of the *atpA* transcript was determined by an *in vitro* capping experiment. Total mitochondrial RNA from the 13F cybrid was labelled by *in vitro* capping and hybridized to the unlabelled antisense probe specific to the 5' region of the *atpA* gene (Figure 7A). The duplexes were treated with RNases and the protected fragments were loaded on a sequencing gel (Figure 7B). No labelled RNA fragments corresponding to the 5' terminus of the *atpA* transcript were obtained. Thus, the 5' end of the *atpA* transcript was not labelled with the capping enzyme guanylyltransferase. This result was expected because the flanking sequences of the 5' end of *atpA* transcript show no similarity to the dicot consensus sequence for transcription initiation (Brown *et al.*, 1991; Binder and Brennicke, 1993). We assume, therefore, that the 3' end of the unstable *orf138* transcript from *Nco2.7/13F* is produced by processing at the 5' end of the *atpA* transcript. The *Brassica orfB* transcript was used as a positive control for the *in vitro* capping experiments

Fig. 6. Analysis of the 3' termini of the *orf138* transcript of *Bam4.8/18S*. (A) RNase protection of the 3' termini of the *orf138* transcript. Autoradiograph of RNase mapping experiments using 20 µg of total RNA isolated from 18S (1 and 2). The control lane corresponds to protection in the presence of 26 µg of yeast tRNA (3). The probe derived by *in vitro* transcription of the *EcoRV*–*EcoRV* fragment has been loaded (P) and is indicated below the figure on the restriction map of *Bam4.8/18S*. The sizes of the protected fragments are indicated. (B) Potential secondary structure formed around the mapped 3' termini of the *orf138* transcript from *Bam4.8/18S*. The 3' ends are indicated by arrows. (C) Northern blot analysis. Mitochondrial RNA was extracted from 18S cybrid, fractionated by electrophoresis, and used for Northern blot analysis. PCR amplified probes are specific to the 3' untranslated region of *orf138* transcript from *Bam4.8/18S* just upstream of the major 3' end (lane 1) or to the t-element (lane 2).

(Figure 7B). When the unlabelled antisense probe derived by *in vitro* transcription of the *HindIII*–*EcoRI* fragment from the *orfB* locus was hybridized to the same *in vitro* capped RNA, two fragments of ~279 and 274 bp were protected and correspond to the 5' termini of the *orfB* transcript predicted by 5' RNase mapping, showing that the capping of mitochondrial RNA was successful.

Analysis of the 3' terminus of the orf138 transcript from Nco2.5/13S. Previous RNase protection mapping of the 3' end of the *orf138* transcripts from *Nco2.5/13S* showed that the 3' end mapped two nucleotides downstream of a potential stem-loop structure (Bonhomme *et al.*, 1992). However, in this previous work, the sizes of the protected fragments were estimated using a DNA ladder and by assuming that the RNA fragments migrate 5–10% slower than the DNA fragments of the same size. Because the

Fig. 7. The 5' end of the *atpA* transcript from *Nco2.7/13F* is a processing site rather than a transcription initiation site. (A) Restriction maps of *Nco2.2* and *Nco2.7/13F*. The transcripts detected from each configuration are indicated by the bent arrows. The regions of the *orfB* and *atpA* loci used as probes in RNase mapping (Map) and *in vitro* capping (Cap) analysis are shown by thick lines below each diagram. (B) *atpA* 5' end analysis: (Map) RNase mapping of the 5' ends of the *atpA* transcripts. The region from the *orf138* coding region to the initiation codon of the *atpA* gene from *Nco2.7/13F* was used as probe (P). M: RNase protection using mitochondrial RNA isolated from 13F cybrid. Cap: RNase protection analysis of capped mitochondrial RNA. Mitochondrial RNA from 13F was radiolabelled by capping and hybridized with the unlabelled probe. After treatment of the heteroduplexes with RNase, the protected fragments were loaded. The DNA sequence around the 5' end of *atpA* gene is shown below and the 5' end corresponding to the processing site is represented by a vertical arrow. (C) Positive control (*orfB* 5' end analysis): (Map) RNase mapping of the 5' ends of the *orfB* transcripts. The transcript derived by *in vitro* transcription of the *HindIII*–*EcoRI* fragment from *orfB* locus was used as probe (P). M: RNase protection using mitochondrial RNA isolated from 13F cybrid. The 5' ends of the *orfB* transcript are indicated by arrows. Cap: RNase protection analysis of capped mitochondrial RNA. *In vitro*-capped mitochondrial RNA from 13F cybrid which is used in the capping experiment with *atpA* transcript, was hybridized with the unlabelled probe from the *orfB* locus. After treatment of the heteroduplexes with RNase, the protected fragments were loaded on the gel. The DNA sequence at the transcription initiation sites is shown below. Transcription initiation sites are presented by bent arrows and the highly conserved sequence in the dicot promoter regions (Brown *et al.*, 1991; Binder and Brennicke, 1993) is boxed.

Fig. 8. Potential secondary structure formed upstream of the 3' end of the *orf138* transcript from *Nco2.5/13S*. The 3' end is indicated by an arrow.

precise location of the 3' ends is important for the decay and processing experiments described below, the 3' terminus was remapped by co-electrophoresis of the RNase-protected fragments with an RNA ladder (data not shown). This showed that the true 3' end lies 20 nucleotides downstream of the potential stem-loop structure (Figure 8).

***In vitro* decay and processing analysis of the 3' region of *orf138* transcripts**

A mitochondrial lysate processes synthetic RNAs corresponding to the 3' region of the *Nco2.5/13S* and *Bam4.8/18S* transcripts. To determine the role of secondary structures found in the 3' region of *orf138* transcripts, an *in vitro* decay and processing system was established. Labelled transcripts containing the 3' region and downstream sequences of *orf138* transcripts were incubated with mitochondrial lysate for 0–45 min as described in Materials and methods.

An RNA precursor of 508 nucleotides corresponding to the 3' region of the *Nco2.5/13S* transcript is rapidly converted into 355- and 153-nucleotide fragments (Figure 9A). Thus, the 3' region of the *orf138* transcript from *Nco2.5/13S* is a substrate for rapid endonucleolytic cleavage in rapeseed mitochondrial lysate. To determine whether the cleavage occurred downstream of the stem-loop structure shown in Figure 8, a second synthetic RNA containing the same upstream sequences but a shorter downstream 3' extension (27 nucleotides) was used in *in vitro* processing. As shown in Figure 9A, the 388 nucleotide substrate is converted to a 355 nucleotide fragment identical to that formed by cleavage of the 508 nucleotide substrate. This strongly suggests that the endonucleolytic cleavage occurs downstream of the stem-loop structure of the *Nco2.5/13S* transcript. A synthetic RNA terminating at this presumed endonucleolytic site co-migrates with the *in vitro*-processed RNA (Figure 9B). Even if the incubation time was reduced to 30 s, no intermediate form with a short 3' extension in comparison with the mature product was seen (Figure 9B). These data suggest that the 3' end of the *orf138* transcript from *Nco2.5/13S* is produced by endonucleolytic cleavage. The 3' end of the *in vitro*

processing product is calculated to be 3 bp upstream of our estimation of the mature *in vivo* 3' end. Thus we suggest that the 3' end of the *in vitro* processing product is similar to the mature *in vivo* 3' end, and given the limits to the resolution of the techniques employed, may well be identical to it.

To determine if the t-element which is present immediately downstream of the 3' end of the *Bam4.8/18S* transcript (Figure 6B) is processed *in vitro*, a synthetic RNA containing the 3' region of the *Bam4.8/18S* transcripts and the t-element sequence not present in the mature transcript was incubated in the mitochondrial lysate. Three major processed products of ~143, 75 and 60 nucleotides were observed (Figure 9C). These sizes are consistent with the predicted molecules which would be obtained by cleavage at the presumed *in vivo* 3' end of the *Bam4.8/18S* transcripts and downstream of the t-element sequence. These results are entirely consistent with the RNase protection data with the natural transcripts.

Our results suggest that the structures found at the 3' region of the *Nco2.5/13S* and *Bam4.8/18S* transcripts act as efficient processing signals that generate the 3' ends found *in vivo*. However, we have not observed *in vitro* processing at the 3' end of the *orf138* transcripts from *Nco2.7/13F*. The rapid degradation of RNA corresponding to the intergenic region between *orf138* and *atpA* may be responsible for the failure to observe a processed product (Figure 9D).

In vitro decay of the 3' region of *orf138* transcripts. To test whether the different 3' regions of the *orf138* transcripts show differential RNA stability in an *in vitro* decay system, synthetic RNAs of the 3' region of *orf138* transcripts terminating at the mature *in vivo* 3' ends were incubated with mitochondrial lysates for 0–60 min. The kinetics for the decay of the three 3' regions show that the most rapidly decaying RNA is, as expected, the 3' region of the *Nco2.7/13F* transcript. The most stable RNA is, as expected, the 3' region of the *Bam4.8/18S* transcript. The 3' region of the *Nco2.5/13S* transcript was moderately degraded (Figure 10). Thus, it is probable that the differences in the steady-state level of *orf138* mRNA between the three configurations involve the divergent 3' regions which play important roles in RNA stability. However, the rate of the degradation of the 3' region of the *Nco2.7/13F* transcript is not as fast as would be predicted by the rarity of *orf138* transcripts from *Nco2.7/13F* *in vivo*. It is possible that some important regulatory proteins are limited in our *in vitro* decay system, or that, *in vivo*, decay is intimately linked to processing. The very fast degradation of RNA corresponding to the intergenic region between *orf138* and *atpA* and containing the processing site (Figure 9D) allowed us to support the latter of these two possibilities.

Discussion

Molecular models for CMS expression in Brassica cybrids

We have previously noted that the expression of the *orf138* gene is correlated with the Ogura cytoplasmic male-sterility in *Brassica* cybrids. Here we show the detailed investigation of the expression of the *orf138* locus which

Fig. 9. *In vitro* processing of precursor RNAs containing the 3' region and downstream sequences of *orf138* transcripts. Labelled transcripts were incubated with rapeseed mitochondrial lysates for 0, 5, 15 and 45 min. The labelled RNA used in each case is indicated below the figure on the restriction map. Times are shown above each panel. The molecular weights are estimated from the migration of *in vitro*-generated transcripts of known size (lane M). To the left are the deduced structures of the precursor and the major processed products. (A) The precursor RNA was a 508- or a 388-nucleotide transcript corresponding to the 3' region of *orf138* transcripts from *Nco2.5/13S* (the 388 nucleotide transcript corresponds to the 5' terminal sequence of the 508 transcript). (B) Lane 1: synthetic RNA terminating at the presumed processing site without incubation with protein; lanes 2, 3, 4 and 5: the 508 nucleotide RNA corresponding to the 3' region of *orf138* transcripts from *Nco2.5/13S* incubated with mitochondrial lysate for 0 s, 30 s, 2 min and 20 min respectively. (C) The precursor RNA is a 278-nucleotide transcript which corresponds to the 3' region of *orf138* transcripts from *Bam4.8/18S*. (D) The precursor RNA is a 414 nucleotide which corresponds to the 3' region of *orf138* transcripts from *Nco2.7/13F*.

Fig. 10. *In vitro* degradation of *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S* 3' RNA. Labelled transcripts of the 3' untranslated regions terminating at the mapped 3' ends of the different *orf138* transcripts were incubated with rapeseed mitochondrial lysates for 0, 5, 20 and 60 min. The decrease in amounts of full length precursor RNA was quantified by phosphorimaging, and the results of two experiments were averaged.

is present in three different organizations, namely, *Nco2.5/13S*, *Nco2.7/13F* and *Bam4.8/18S* in the sterile 13S, fertile 13F and sterile 18S cybrids respectively. A model to explain the expression of the different *orf138* loci is shown in Figure 11. Although there are probably other mechanisms involved in the expression of *orf138*, RNA stability has an important role. The 3' region of the transcripts clearly functions as *cis*-acting elements that determine processing and stability.

tRNA processing helps generate the mature termini of *orf138* mRNAs

Three of the five processing events mapped in this work occur immediately 5' or 3' of a bona fide tRNA or t-element. The 5' terminus of all *orf138* mRNAs is generated by endonucleolytic cleavage 3' to the tRNA^{fMet} sequence. The major and minor 3' termini of the *orf138* mRNA in the cybrid 18S are respectively generated by endonucleolytic cleavages immediately 5' and 3' of a t-element. Similar t-element sequences have been found in wheat mitochondrial DNA (Joyce *et al.*, 1988). *In vitro* processing of these t-element sequences was observed in wheat mitochondrial extract (Hanic-Joyce *et al.*, 1990). Processing 5' to a tRNA or t-element sequence is likely to involve RNase P (Marchfelder and Brennicke, 1993), but the enzyme responsible for 3' cleavage is presently unknown. A 3' endonucleolytic cleavage rather than 3'→5' exonuclease processing has already been suggested for the production of the 3' end of several plant mitochondrial tRNAs and such cleavages have been observed *in vitro* (Hanic-Joyce and Gray, 1990; Hanic-Joyce *et al.*, 1990; Binder and Brennicke, 1993; Remacle and Maréchal-Drouard, 1996). In addition, the 5' termini of other plant mitochondrial RNAs may be determined by cleavage 3' of tRNAs. For example, *trnfM* has been shown to be closely linked and co-transcribed with other genes: *atp6* in radish (Makaroff *et al.*, 1989) and fertile *B.napus* (Singh

and Brown, 1991), and *rrn18* in wheat (Gray and Spencer, 1983). These data suggest that although not a general rule, processing of tRNA sequences may often be involved in mitochondrial gene expression in plants. In mammalian mitochondria most monocistronic RNAs are produced by endonucleolytic cleavage of the primary transcripts upstream and downstream of tRNA sequences (Clayton, 1984).

A stabilizing role for the 3' region of the *orf138* transcripts

Our data show a correlation between the steady-state mRNA level of the *orf138* locus and the rate of *in vitro* decay of the 3' region of the *orf138* transcripts. Processing of the 3' regions of the *Nco2.5/13S* and *Bam4.8/18S* transcripts occurs downstream of secondary structures which might increase the stability of the upstream mRNAs by increasing resistance to exonucleolytic degradation from the 3' end. Such secondary structure-forming inverted repeat sequences have been found downstream of the coding regions of many plant mitochondrial genes (Bland *et al.*, 1986; Schuster *et al.*, 1986; Rothenberg and Hanson, 1987; Wahleithner and Wolstenholme, 1988; Wissinger *et al.*, 1988; Gualberto *et al.*, 1990; Macfarlane *et al.*, 1990; Saalaoui *et al.*, 1990; Kaleikau *et al.*, 1992; Liu *et al.*, 1992). It has been suggested that the 3' secondary structures act either as transcriptional terminators (Schuster *et al.*, 1986), processing signals (Bland *et al.*, 1986; Wissinger *et al.*, 1988; Macfarlane *et al.*, 1990) or stabilizing signals (Liu *et al.*, 1992). Our *in vitro* studies of the 3' regions of the *Nco2.5/13S* and *Bam4.8/18S* transcripts show that these sequences play roles in both processing and stabilizing the RNA. Results consistent with ours have been obtained by studying the potential role of the stem-loop structures found at the 3' end of the pea mitochondrial *atp9* transcript in the accompanying paper (Dombrowski *et al.*, 1997). It has been shown that the 3' inverted repeats act as mRNA processing elements, but do not terminate transcription in a pea *in vitro* system (S.Dombrowski, A.Brennicke, S.Binder, submitted). These data are consistent with those on the 3' stem-loop regions of chloroplast transcripts, which also have functions in processing and stabilization (Adams and Stern, 1990; Stern and Gruissem, 1987; Stern *et al.*, 1989, 1991; Stern and Kindle, 1993).

The 3' region of *Nco2.5/13S* is also present in the 3' region of the normal copy of the *orfB* gene in *Brassica* cybrids (locus *Nco2.2* in Figure 7) and the 3' untranslated region of *Bam4.8/18S* is also found in the 3' untranslated region of *Brassica orf577a* (Handa *et al.*, 1996). The t-element found in the 3' region of *Bam4.8/18S* shows high similarity to a sequence present in the 3' region of the *nad5* gene in *Vicia faba* (vfanad5de.gb_pl, accession L36946). It is clear that the 3' sequences studied in this paper are present downstream of other expressed genes and may play similar roles in processing and stabilizing their transcripts.

In this paper, we show that the *orf138* and *atpA* coding regions are co-transcribed from *Nco2.7/13F* and that the 5' end of the *atpA* transcript is a processing site rather than a transcription initiation site. We assume that endonucleolytic cleavage between the *orf138* and *atpA* coding sequences constitutes the initial step for the degradation

Fig. 11. Proposed model for the expression of the *orf138* locus. The *orf138* locus is transcribed from a transcription initiation site upstream of *trnfM*. Transcripts from the three configurations are processed downstream of *trnfM* by 3' endonucleolytic cleavage. The transcripts from the *Nco2.5/13S* and *Bam4.8/18S* transcripts are processed 3' to the inverted repeat and upstream of the t-element respectively. The *Nco2.7/13F* transcript is cleaved at a processing site between the *orf138* and *atpA* coding regions. The processed transcripts from *Nco2.5/13S* and *Bam4.8/18S* are stabilized by their 3' regions and give rise to a large amount of *orf138* protein, while the processed *orf138* transcript from *Nco2.7/13F* is rapidly degraded, so the *orf138* protein is not accumulated. The presence of large amounts of the *orf138* protein in mitochondria from 13S and 18S causes male-sterility.

of *orf138* mRNA and for the production of the monocistronic *atpA* mRNA. The 3' region of the upstream *orf138* mRNA lacks obvious stem-loop structures and presumably is not stabilised against rapid degradation. Interestingly, no transcript is detected from the sequence upstream of the processing site of the normal *atpA* gene which has identical upstream sequences to the *Nco2.7/13F* *atpA* gene (data not shown).

The involvement of proteins in stabilizing mRNAs

The calculated free energies of the stem-loop structures found at the 3' termini of *orf138* transcripts from *Nco2.5/13S* and *Bam4.8/18S* are not particularly impressive and do not obviously correlate with the *in vitro* stability of these RNAs or the steady-state mRNA level *in vivo*. It is likely, therefore, that the stabilizing effects of these 3' regions involve specific interactions with proteins. Preliminary UV cross-linking experiments show that synthetic RNA containing the 3' region of the *orf138* transcript from *Bam4.8/18S* binds to a protein present in the rapeseed mitochondrial lysate. Further investigation is required to determine the degrading or stabilizing proteins which interact with the 3' termini of the *orf138* transcripts. Although little is currently known about the control of messenger RNA stability and processing in plant mitochondria, *Brassica* cybrids carrying different con-

figurations of the *orf138* gene are ideal material for making significant advances in this domain.

Materials and methods

Plant material

The *B.napus* cybrids examined in this report resulted from protoplast fusion between a male-sterile *Brassica* carrying Ogura cytoplasm and the *B.napus* spring variety Brutor as described in Bonhomme *et al.* (1991) and Grelon *et al.* (1994). *Brassica napus* cv Brutor plants were used for the preparation of mitochondrial lysate. The plants were grown in the greenhouse.

Purification of mitochondria

Ten grams of buds of greenhouse-grown *Brassica* plants were ground in extraction buffer [0.4 M sucrose, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM β -mercaptoethanol, 0.5% bovine serum albumin (BSA)]. The homogenate was filtered through four layers of Miracloth (Calbiochem). The filtrate was centrifuged by briefly accelerating to 7500 r.p.m. in an A 6.14 rotor (Kontron), and the supernatant was centrifuged at 11 000 r.p.m. for 15 min in an A 6.14 rotor. The crude mitochondrial pellet was resuspended with a brush in resuspension buffer (0.4 M mannitol, 50 mM Tris-HCl pH 7.5, 0.5% BSA), and layered on Percoll (Pharmacia) density gradients consisting of 4.5 ml of 60%, 5.2 ml of 29%, and 3 ml of 21% Percoll in 0.25 M sucrose, 50 mM Tris-HCl pH 7.5. The gradient was centrifuged at 9500 r.p.m. for 45 min in an A 8.24 rotor (Kontron). The mitochondria were recovered from the 29–60% interface, diluted three times with resuspension buffer, pelleted at 12 000 r.p.m. for 15 min, and used either in run-on transcription assays or in the *in vitro* degradation and processing system.

Isolation of RNA and RT-PCR experiments

Total RNA was extracted from buds by the method of Dean *et al.* (1985). For mitochondrial RNA isolation, mitochondria were partially purified from buds by differential centrifugation, and then mitochondrial RNA was extracted by the method of Dean *et al.* (1985). RT-PCR experiments were carried out as described in Monéger *et al.* (1994).

Slot blot and hybridization analysis

RNA was separated by electrophoresis and transferred to Hybond-N (Amersham) membrane according to the method of Monéger *et al.* (1994). Total DNA or total RNA was denaturated and immobilized on a slot blot using Hybond-N membrane. The hybridization was performed overnight at 42°C in 50% deionized formamide, 5× SSPE (0.9 M NaCl, 5 mM EDTA, 50 mM sodium phosphate, pH 7.4), 5× Denhardt's solution (Sambrook *et al.*, 1989), 0.5% N-lauryl-sarcosine and 40 µg/ml denatured salmon sperm DNA. The membrane was washed once for 30 min in 2× SSC, 0.5% N-lauryl-sarcosine and 0.2% Na₄P₂O₇ at 65°C and once for 15 min in 0.2× SSC, 0.5% N-lauryl-sarcosine and 0.2% Na₄P₂O₇ at room temperature.

Preparation of synthetic RNAs, primer extension, RNase protection and run-on transcription analysis

In vitro RNA probes prepared from linearized plasmid DNA and primer extension experiments were carried out according to Bonhomme *et al.* (1992). For the preparation of labelled transcripts terminating at the *in vivo* mature mRNA 3' end, PCR experiments were performed with clones of 3' region-containing fragments. One primer was complementary to the plasmid sequence and the other was complementary to the *in vivo* mature mRNA 3' end. The PCR products were then used for *in vitro* transcription as for linearized plasmid DNA.

RNase protection experiments were carried out as described in Liu and Bergold (1994) except that the ribonuclease treatment was carried out either at 30°C for 60 min or at 25°C for 10 min.

For run-on assays, mitochondria were purified as described above, and labelled mitochondrial transcripts were synthesized by the method of Mulligan *et al.* (1991).

In vitro capping experiments and kinase reactions

Mitochondrial RNAs were capped by guanylyltransferase as described by Mulligan *et al.* (1988). *In vitro* capped mitochondrial RNAs were used either in RNase protection experiments as described above or as a probe for Southern blot experiments. For the kinase reactions, *Brassica* cybrid mitochondrial RNA was labelled with [γ -³²P]ATP by the method of Remacle and Maréchal-Drouard (1996).

In vitro decay and processing of RNA

Purified mitochondria from normal *B.napus* cv Brutor (100 µg of proteins) were lysed in degradation buffer containing 30 mM Tris-HCl pH 8, 40 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.2 mM GTP, 0.5 mM Pefabloc (Interchim) and 0.1% Triton X-100. RNA substrates generated by *in vitro* transcription (30 000 c.p.m.) were added to the ice-cold mitochondrial lysates in a final volume of 60 µl. The reaction mixture was incubated at 28°C, and 13 µl aliquots were removed at various time points. Decay and processing reactions were terminated by placing samples in a solution (100 µl) containing 0.5% SDS and 12.5 mM EDTA. After phenol extraction, RNA was precipitated and analysed on 6% polyacrylamide gels. Samples representing time 0 min were taken just after the RNA substrates were added to the ice-cold mitochondrial lysates.

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