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Characterization of radish mitochondrial *atpA:* influence of nuclear background on transcription of *atpA*-associated sequences and relationship with male sterility

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Abstract

We have previously shown that the mitochondrial gene *atpA*, encoding the α subunit of F_1 ATP synthase, is associated with DNA rearrangements and nuclear-specific transcript patterns in the male-sterile cytoplasm of Ogura radish. Here we present a detailed characterization of this gene from both the normal (fertile) and Ogura (male-sterile) cytoplasms of radish to determine if it is involved in Ogura cytoplasmic male sterility. The normal and Ogura radish *atpA* loci are virtually identical for 3.8 kb, including a 507 codon open reading frame whose product is approximately 92% identical to other plant ATPA polypeptides. Rearrangement breakpoints have been identified 613 bp 5′ and 1663 bp 3′ to the *atpA* coding region. The 5′ rearrangement breakpoint is located within a repeated sequence that has been associated with other rearrangement events in radish mitochondria. The previously identified transcript difference results from transcription originating upstream of this rearrangement site. Although the presence of this transcript is affected by nuclear background, analyses in several different sterile and fertile nuclear backgrounds indicate that the presence of this transcript is not strictly correlated with male sterility. In addition, normal levels of ATPA polypeptide are present in sterile plants containing the Ogura cytoplasm.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that is characterized by a plant's inability to produce functional pollen. Male-sterile cytoplasms have long been of interest because of their use in hybrid seed production; however, they also provide a useful system in which to study mitochondrial function and nuclear-mitochondrial interactions. There are numerous examples of alterations in mitochondrial DNA (mtDNA) structure and mitochondrial RNA (mtRNA) and protein patterns associated with CMS plants [reviewed in 21, 22]. Nuclear loci that suppress these mitochondrial alterations and restore fertility to CMS cytoplasms have also been identified.

The Ogura cytoplasm of radish (*Raphanus*

sativus) was the first example of CMS in a crucifer (family Brassicaceae). Radish plants containing the Ogura cytoplasm develop normally until early microspore formation when a collapse of the tapetal tissue leads to microspore degeneration [29]. Three nuclear loci have been identified in European cultivars of radish that may be involved in fertility restoration of the Ogura cytoplasm in *Brassica napus* [14]. However, Pelletier *et al.* [32] have suggested that the male sterility observed for the *B. napus*-Ogura cytoplasm combination may be the result of both mitochondrial alterations (observed in a radish nuclear background) and nuclear-mitochondrial incompatibilities (alloplasmic male sterility). Therefore, it is not clear how many nuclear loci are necessary to restore fertility to the Ogura cytoplasm in radish.

The mitochondrial genome of Ogura radish is highly rearranged relative to, and contains sequences not present in, the normal radish genome [25]. In addition, altered transcript patterns have been identified for three genes: *atpA*, *atp6*, and *coxI*. Of these genes, only the *atpA* transcript pattern is affected by nuclear background. In order to identify the mitochondrial alteration(s) associated with Ogura CMS, we have characterized the Ogura mitochondrial genome further, including the detailed analyses of these three genes. Altered *atp6* transcript patterns are the result of a complex series of rearrangements that have resulted in an Ogura-specific transcriptional unit containing a 105 codon open reading frame as the first gene of a bicistronic mRNA. In addition, multiple nucleotide differences in the Ogura *atp6* 5′ flanking region and coding sequence are predicted to eliminate normal translation of *atp6* [24]. In contrast, characterization of the normal and Ogura radish *coxI* loci indicates that *coxI* is not involved in Ogura CMS and that the observed differences in *coxI* transcript patterns are the result of a rearrangement that has occurred in normal radish (Makaroff, Apel and Palmer, unpublished).

We report here a detailed analysis of the *atpA* locus from both normal and Ogura radish. The previously identified transcript difference is the result of transcription that originates upstream of a 5′ rearrangement breakpoint at Ogura *atpA*. Analysis of Ogura *atpA* transcript patterns in several different nuclear backgrounds indicates that while the presence of this transcript is dependent on nuclear background, it is not strictly correlated with male sterility. Furthermore, normal levels of ATPA are present in sterile and fertile plants containing the Ogura cytoplasm, indicating that *atpA* is not causally related to CMS.

Materials and methods

Plant material

Raphanus sativus cv. Scarlet Knight (wild-type radish) was the source of the normal radish cytoplasm. The mitochondrial genome of Scarlet Knight exhibits the same restriction enzyme profile as those of three other commonly used male-fertile radish cultivars ([30], Makaroff and Palmer, unpublished) and thus is used to represent what we term the 'normal' cytoplasm of radish. The rapid-cycling radish line CrGC15 (R1rr; obtained from the Crucifer Genetics Cooperative) was the primary source of the Ogura cytoplasm. Three other sources of the Ogura cytoplasm used in this study were: 1) an open-pollinated white radish line (Iwate) that segregates approximately 50 : 50 for fertile and sterile plants; 2) a *B. napus* line containing the Ogura cytoplasm (male-sterile) (this and the preceding line were obtained from Dr. J. Imamura, Plantech Research Institute, Japan); and 3) a second open-pollinated white radish line (5241) that segregates for fertile and sterile plants (50 : 50) (obtained from Dr. S. Magnuson, Harris Moran Seed Co., CA). Sterile and fertile (nuclear-restored) plants containing the Ogura cytoplasm were distinguished by their ability to produce pollen.

Isolation and labeling of nucleic acids

Mitochondrial DNA was isolated from green leaves of both normal radish and plants contain-

ing the Ogura cytoplasm (CrGC15, Iwate and *B. napus*) by the DNase I procedure [18]. Mitochondrial RNA was isolated in the presence of aurintricarboxylic acid from mitochondria purified by differential centrifugation from green leaves (normal radish) and green leaves and flowers (Ogura cytoplasm) [38]. Plasmid DNA was isolated using the alkaline lysis procedure [3] and purified over CsCl gradients as necessary. Probes used in S1 nuclease mapping and northern and Southern hybridization experiments were labeled using common techniques [27], including primer extension of single-stranded DNA, nick translation and 5′- or 3′-end labeling with T4 kinase or Klenow fragment, respectively, followed by purification by gel electrophoresis. Single-stranded molecules for the 277 bp *Eco*RI and 228 bp *Bgl*II-*Spe*I fragments were isolated on strand separation gels [27] and their identity (coding vs noncoding strand) determined by northern analysis.

DNA sequence analysis

Normal radish *atpA* was previously localized to sequences spanning *Sal*I fragments of 3.4 and 13.6 kb [25]. Most of the normal *atpA* locus was subcloned as a 2.8 kb *Sal* I-*Hin*dIII fragment from the previously isolated 3.4 kb *Sal* I fragment [31]. The 5′ coding and flanking regions were isolated as a 1.2 kb *Bam*HI fragment by colony hybridization screening [27] of a normal radish mtDNA library with a 0.4 kb *Bam*HI-*Sal* I fragment internal to Ogura *atpA* (Fig. 1). The Ogura radish *atpA* locus was subcloned as 1.25 kb *Nru* I-*Sal* I and 3.45 kb *Sal* I-*Bam*HI fragments from previously isolated 12.0 kb and 9.6 kb *Sal* I fragments, respectively [25].

Standard techniques were used in the construction and analysis of recombinant clones [27]. Dideoxy sequencing [34] of exonuclease III- [11] and restriction enzyme-deleted clones with vector- and *atpA*-specific oligonucleotides was conducted as described [24]. DNA sequences were analyzed on a Sun Microsystems, Inc. minicomputer using the Eugene program (Department of Cell Biology, Baylor College of Medicine, Houston, TX).

S1 nuclease mapping

Transcript mapping experiments were conducted using the procedure of Berk and Sharp [2]. RNA (10 μg) was co-precipitated together with a DNA probe (Fig. 4), resuspended in 20 μl of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide), denatured at 80 °C for 5 min, and allowed to anneal for 12–16 h at 45–56 °C (the GC content of the fragment determined the annealing temperature). Samples were treated with 300 μl of a buffer containing 250 mM NaCl, 1 mM $ZnSO_4$, 30 mM potassium acetate, pH 4.5 and 5% (v/v) glycerol for 60 min at 37 °C. The samples were then phenol-extracted, ethanol-precipitated, resuspended in denaturing stop dye and electrophoresed on both 5% polyacrylamide/8 M urea gels and high-resolution 6% polyacrylamide/8 M urea gels next to ^{32}P-labeled Bluescript KS digested with

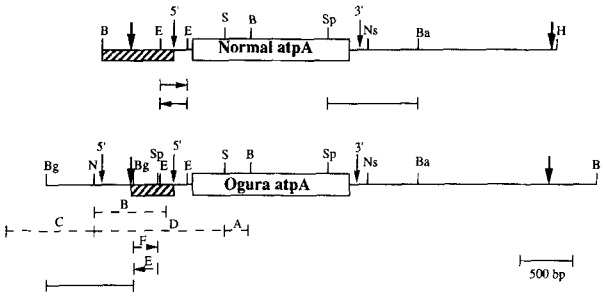

Fig. 1. Organization of the *atpA* loci from the normal and Ogura cytoplasms of radish. The *atpA* locus of normal radish (top) is shown aligned with that of Ogura radish (bottom). Open boxes represent the *atpA* coding regions; shaded boxes represent sequences present elsewhere in the radish genomes. Rearrangement breakpoints are marked with heavy vertical arrows. Fragments used as probes in nuclease mapping experiments and northern hybridizations are shown as solid and dashed lines, respectively. Single-stranded probes are shown as horizontal arrows. Transcript termini are denoted with light vertical arrows. Selected restriction sites, which were used in the construction of probes and fusion proteins, are shown as: B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; Ns, *Nsi*I; S, *Sal*I; Sp, *Spe*I.

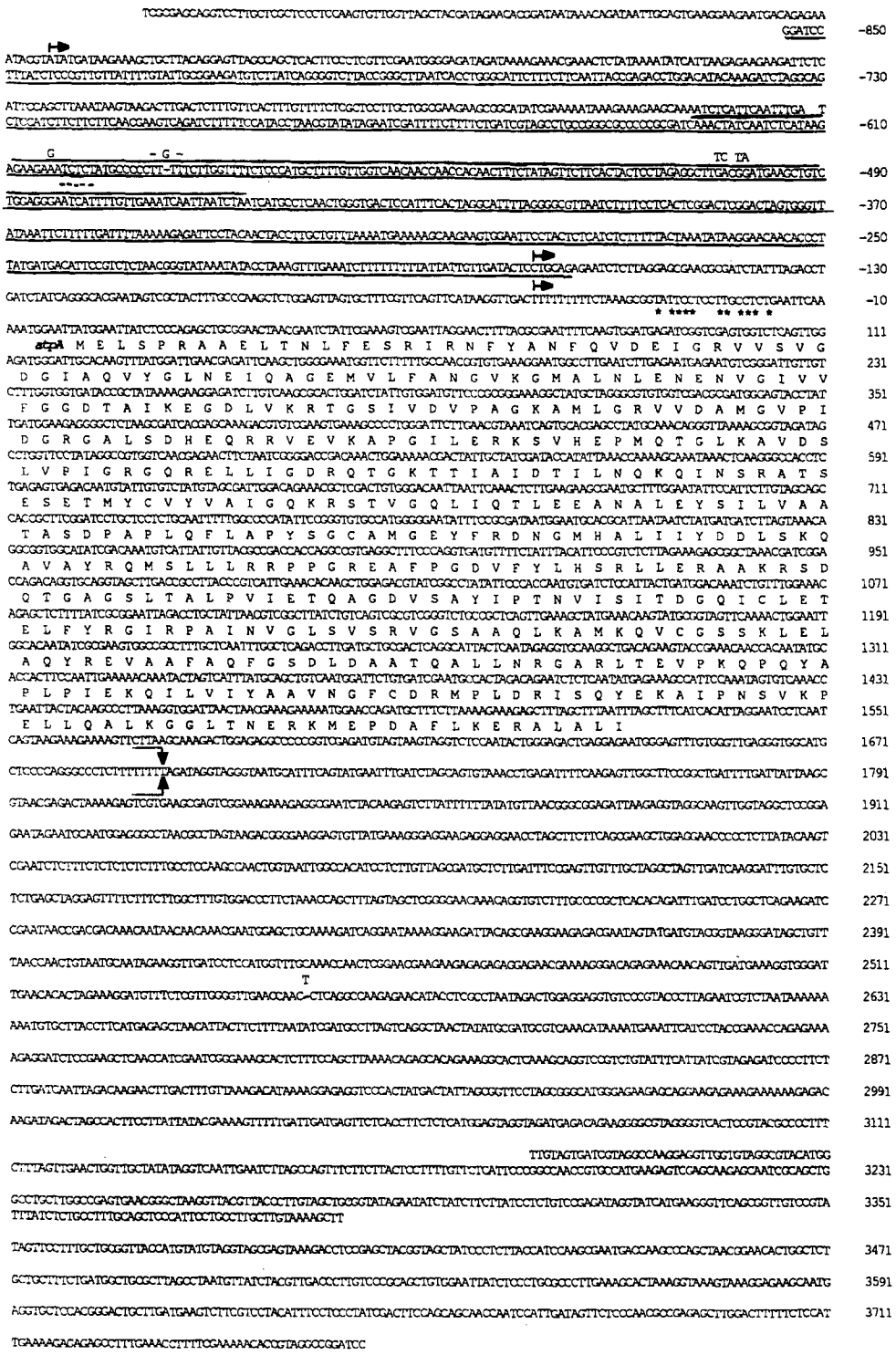

Fig. 2. Nucleotide and derived amino acid sequences of the *atpA* loci from normal and Ogura radish. The Ogura sequence (top line) is aligned with the normal sequence (bottom line). Where the two sequences are identical only the normal sequence is shown. Insertions/deletions relative to normal *atpA* within the aligned regions are represented by a dash (–). Numbering of nucleotides is from the predicted translation start of *atpA*. A 689 bp sequence present 5′ to normal radish *coxI–2* is underlined; a 175 bp sequence present 5′ to the Ogura *orf105–atp6* locus is overlined. Nucleotides complimentary to the 18S rRNA, allowing for G-U

*Hin*fI and dideoxy sequencing ladders, respectively.

Northern and Southern blot analysis

Conditions for the electrophoresis, transfer and hybridization of RNA (7.5 μg/lane) to ^{32}P-labeled probes were as described previously [26]. Ridosomal RNAs and *Hae* III fragments of phage ΦX174 were used as size standards. Probe used in hybridization experiments are described in Fig. 3. Standard techniques were used in the construction of Southern blots and their hybridization to nick-translated probes [27].

Antibody production and western analysis

A 993 bp *Bam* HI-*Nsi* I fragment of normal radish *atpA* (nucleotides 720 to 1713; Figs 1 and 3) was fused in-frame to *Escherichia coli lacZ* in the vector pUR290 [31] to produce a β-galactosidase fusion protein containing the carboxy-terminal 262 amino acids of radish *atpA*. The fusion protein was overproduced in the presence of 1.0 mM IPTG, purified twice on 10% SDS-polyacrylamide gels [19] and used to raise antibodies in New Zealand White rabbits following standard procedures [12]. A primary injection of protein (200 μg) in Freund's complete adjuvant was given, followed by two boosters (200 μg protein each in Freund's incomplete adjuvant) at six-week intervals. Serum was collected to weeks after the second boost. Prior to use, serum containing ATPA antibodies was treated with acetone-extracted proteins prepared from *E. coli* cells containing the plasmid pUR290. Preimmune serum when used in western hybridizations did not cross-react with the 55 kDa mitochondrial protein (data not shown). Antibody to maize *coxII* and the spinach chloroplast 32 kDa protein was kindly provided by C. S. Levings III and N. Bowlby, respectively.

Mitochondria were isolated from radish leaves by differential centrifugation and purified over Percoll gradients [20]. Mitochondrial proteins were separated on 12.5% SDS-polyacrylamide gels [19] and transferred to nitrocellulose using the procedure of Towbin [39]. Western blots were treated with primary antibodies overnight and developed using a Bio-Rad Immuno-Blot Goat-Anti-Rabbit Alkaline Phosphatase assay kit under conditions recommended by the supplier. Protein content was determined as previously described [23].

Results

Rearrangements and novel sequences are associated with atpA in Ogura radish

The nucleotide sequence of a 4.1 kb segment of normal radish mtDNA that contains the *atpA* locus is shown in Fig. 2. Present in this sequence is a 507 codon open reading frame capable of encoding a 55 113 Da polypeptide, identified as *atpA* on the basis of DNA sequence identity. The derived amino acid sequence is 91–94% identical to that of maize [6], pea [28], *Oenothera* [35] and tobacco [7]. If however the *atpA* transcript is edited, as has been observed for several other plant mitochondrial genes [8, 11, 37], then the amino acid similarity may be different.

The nucleotide sequence of a 4.7 kb segment of Ogura radish mtDNA containing the *atpA* locus is shown aligned with that of normal radish in Fig. 2. The two loci are virtually identical (99.8%) for 3.8 kb. Six nucleotide substitutions and five insertions/deletions are present in the common region. Rearrangement breakpoints between the two loci are located 613 bp 5′ and 1663 bp 3′ to the *atpA* coding region. The 5′ rearrangement breakpoint is located within a 689 bp sequence (nucleotides − 855 to − 166) that is repeated elsewhere in the genome. Filter hybridizations indicate that part or all of this sequence is present at

basepairing, are indicated with an asterisk (*) under the corresponding sequence. Major 5′ transcript termini are indicated with horizontal arrows and 3′ transcript termini are indicated with vertical arrowheads (above the sequence for Ogura radish and below the sequence for normal radish).

least four and six times in the normal and Ogura radish genomes, respectively (Makaroff, Apel and Palmer, unpublished). This dispersed repeat appears to be involved in homologous recombination events that generated a submolar form of the *coxI* gene. The boundaries of this repeat have been assigned based on a comparison with the *coxI*-associated repeat element. The sequences of additional members of this repeat family have not been determined, therefore the sizes of these repeat elements relative to the *atpA* repeat may be different from that defined by the *atpA*/*coxI* comparison.

From the 5′ end of the sequenced region to nucleotide − 613 the two *atpA* loci are completely different. Southern hybridization experiments using Ogura mtDNA fragments from this region as probes indicate that this sequence is essentially absent from normal radish (data not shown). Multiple weak hybridization signals are, however, detected in both normal and Ogura mtDNAs, suggesting that this region contains one or more short, repeated sequences. Computer analysis of the Ogura mtDNA sequence from − 1955 to + 1 did not identify any open reading frames or similarity with known genes. Southern hybridizations indicate that this sequence is not derived from chloroplast DNA (data not shown), and therefore, the origin of this DNA sequence is unknown. These results are in agreement with earlier findings that the Ogura 12.0 kb *Sal* I fragment is, for the most part, not present in normal radish [6].

Nuclear background affects the processing of atpA-related transcripts in Ogura radish

A series of transcript mapping experiments was conducted in order to characterize a previously identified 2700 nt *atpA* transcript, which is associated with male sterility in rapid-cycling radish lines containing the Ogura cytoplasm (CrGC15) [25], and also to ascertain whether this transcript is causally related to Ogura CMS.

In order to determine the relationship between the 2700 nt transcript and the Ogura *atpA* locus, northern hybridizations were performed using mtRNA isolated from normal radish and fertile and sterile CrGC15 radish plants containing the Ogura cytoplasm. A 400 bp *Sal* I-*Bam* HI fragment, internal to the *atpA* coding region and labeled on the coding strand, hybridized to a single 1800 nt transcript in normal radish and fertile CrGC15 mtRNA preparations, but to transcripts of 1800 nt and 2700 nt in sterile CrGC15 mtRNA (Fig. 3A). Experiments using a

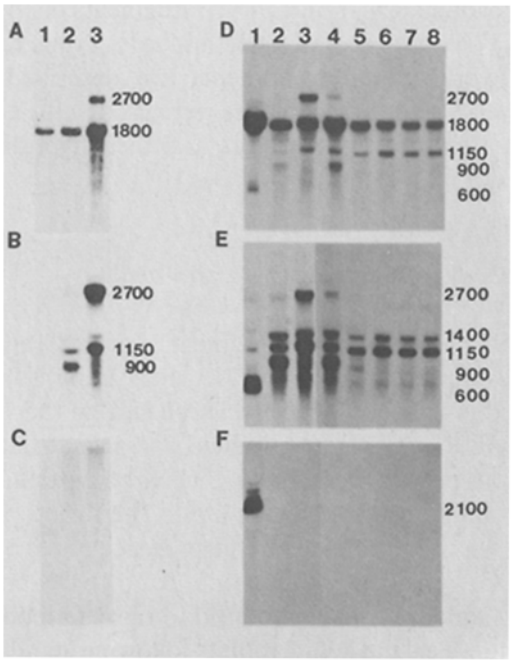

Fig. 3. Northern hybridization analysis of *atpA*. MtRNAs (7.5 μg each) are from normal radish (1), fertile (2) and sterile (3) CrGC15 radish, fertile (4) and sterile (5) 5241 radish, fertile (6) and sterile (7) Iwate radish, and *B. napus* (8) plants containing the Ogura cytoplasm. Filters were probed with the following fragments (see Fig. 1 for probe locations): a 400 bp *Sal* I-*Bam* HI fragment internal to the *atpA* coding region and end-labeled at the *Sal* I site with Klenow fragment and α-^{32}P-dATP (panel A); a primer extension-generated fragment containing 766 nt of coding-strand DNA from the Ogura *atpA* 5′ flanking region (nucleotides − 959 to − 184) (panel B); a nick-translated plasmid containing a 2.0 kb *Hind* III-*Nru* I fragment from the Ogura *atpA* 5′ flanking region (panel C); a nick-translated plasmid containing a 1.25 kb *Nru* I-*Sal* I fragment from the Ogura *atpA* 5′ flanking and coding region (panel D); a 228 nt single-stranded, *Bgl* II-*Spe* I (coding-strand) fragment labeled with T4 polynucleotide kinase and γ-^{32}P-ATP and purified by strand separation (panel E), and a 228 nt single-stranded *Bgl* II-*Spe* I (noncoding-strand) fragment, which is the complement of the probe in panel E (panel F). Sizes are shown in nucleotides.

probe from the Ogura *atpA* 5′ flanking region (nucleotides −950 to −184) identified the 2700 nt transcript as well as Ogura-specific transcripts of 1150 nt and 900 nt, but not the 1800 nt transcript (Fig. 3B). A 2 kb probe containing sequences 5′ to the *Nru*I site (nucleotide −950) failed to detect any transcripts (Fig. 3C). These results indicate that the 2700 nt transcript originates from the Ogura *atpA* locus and also show that the 2700 nt and 1800 nt transcripts are derived from sequences 3′ to nucleotides −950 and −184 respectively.

We next wished to investigate the affect of nuclear background on the expression of the 2700 nt transcript and to determine if the presence of this transcript is strictly correlated with the male-sterile phenotype. A 1282 bp *Nru*I-*Sal* I fragment containing 949 bp and 333 bp of Ogura *atpA* 5′ flanking and coding regions, respectively, was used to probe northern blots of mtRNA isolated from the normal radish cytoplasm and from the Ogura radish cytoplasm in the presence of seven different nuclear backgrounds (Fig. 3D). As expected, the 1800 nt *atpA* transcript is present in every mtRNA preparation. The 2700 nt transcript is detectable in mtRNA isolated from sterile CrGC15 radish plants (lane 3) and at low levels in fertile (nuclear-restored) 5241 radish plants (lane 4). This transcript is not observed in mtRNA preparations from sterile plants containing the Ogura cytoplasm and two different white radish nuclear backgrounds (lanes 5 and 7), or from sterile plants containing the Ogura cytoplasm and the *B. napus* nucleus (lane 8). This result indicates that while the presence of the 2700 nt transcript is affected by nuclear background, its presence is not strictly correlated with the sterile phenotype.

Single-stranded probes from the Ogura *atpA* 5′ flanking region were then used to further delimit and characterize the *atpA* transcripts. A probe from the 5′ flanking region, corresponding to the *atpA* coding strand (nucleotides −604 to −376), revealed a complex transcript pattern (Fig. 3E). A 600 nt transcript not present in any of the Ogura mtRNA preparations is detected in normal radish mtRNA. Present in all of the Ogura mtRNA preparations are transcripts of 1400 nt and 1150 nt. A third transcript (900 nt) is abundant in mtRNA preparations from fertile plants containing both the CrGC15 nucleus (lane 2) and the 5241 nucleus (lane 4). This transcript was not detected in fertile plants containing the Iwate nucleus or in any of the sterile plants. When the same region of the opposite strand (noncoding) was used as the probe in northern hybridizations, two transcripts (2100 and 2150) were identified in normal radish mtRNA (Fig. 3F). No hybridization signals are observed for any of the Ogura mtRNAs with this probe. All of the additional transcripts identified with the 228 bp, strand-specific, probes are also selected with the 1282 bp, double-stranded, *Sal* I-*Nru*I probe used in Fig. 3D upon prolonged autoradiographic exposure (data not shown). In contrast to the 2700 nt transcript, the 2150, 2100, 1400, 1150, 900 and 600 nt transcripts appear to be the result of transcription originating at other regions of the genome and are therefore not directly related to *atpA*. Consistent with the theory, probes internal to the *atpA* coding region and 5′ to the *Nru*I site (nucleotide −994) do not detect these transcripts (Fig. 3A and 3C). In addition, the transcripts exhibit low-level hybridization signals when the *Sal* I-*Nru*I fragment is used as a probe, but strong signals when small, strand-specific probes, corresponding to a repeat element present several times in both normal and Ogura mtDNAs, are used. Further experiments are necessary to determine the location of these additional transcribed regions and whether they have any coding capacity.

The 5′ and 3′ transcript termini of the 1800 and 2700 nt transcripts were determined by S1 nuclease mapping experiments. The 3′ transcript terminus of *atpA* in normal radish an sterile and fertile Ogura CrGC15 radish was mapped using a 739 bp end-labeled *Spe*I-*Bal* I fragment (Fig. 1). One major protected fragment of 358 nucleotides was obtained from all three mtRNAs (Fig. 4D). This corresponds to a 3′ transcript terminus at nucleotide 1693, 172 nt downstream from the *atpA* coding region (Fig. 2).

The 5′ terminus of the 1800 nt transcript was

mapped using both strands of a 277 nt *Eco*RI fragment (– 295 to – 18 and – 291 to – 14). The coding strand yields abundant protected fragments of 160 and 277 nt when used as a probe with the three RNAs described above (Fig. 4A). These correspond to a major transcript terminus at – 172 and fully protected probe. A major 5′ transcript terminus at – 172 and a 3′ terminus at 1693 predict an *atpA* transcript of 1865 nt; this correlates well with the 1800 nt size estimate from northern hybridizations. While we can not eliminate the possibility that the presence of fully protected probe is the result of transcripts originating 5′ to the *Eco*RI site (– 295), this seems unlikely given the presence of one predominant *atpA* transcript (Fig. 3A) and the fact that a probe spanning the region from nucleotides – 950 to – 185 fails to hybridize to the 1800 nt *atpA* transcript (Fig. 3B). Control lanes indicate that the presence of these bands is not the result of incomplete nuclease. Rather we propose that transcripts corresponding to repeated sequences present elsewhere in the genome are responsible for complete protection of the fragment. If this is true, then other copies of the repeat must be larger than the region in common to the *atpA* and *coxI* loci. Further characterization of these additional repeat elements is required to confirm this possibility. Control experiments using the noncoding strand of the *Eco*RI fragment as the probe resulted in no protection by the Ogura mtRNAs but low levels of fully protected probe with normal radish mtRNA (Fig. 4B). This is compatible with the finding that the noncoding strand of a repeat-containing fragment hybridizes to transcripts in normal radish mtRNA but not Ogura radish mtRNA (Fig. 3F).

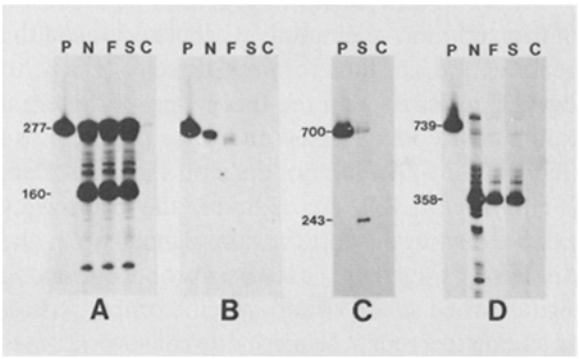

Fig. 4. Nuclease mapping of the 5′ and 3′ termini of the normal and Ogura radish *atpA* transcripts. Autoradiographs of nuclease mapping experiments using mtRNA (10 μg) isolated from normal radish (N), and sterile (S) and fertile (F) CrGC15 plants are shown. Control lanes (C) contained 20 μg of yeast tRNA. Probes (P) used were as follows: panel A, 277 nt single-stranded *Eco*RI (coding strand) labeled with γ-^{32}P-ATP and T_4 polynucleotide kinase and purified by strand separation; panel B, the noncoding strand of the fragment in panel A; panel C, 700 bp *Bgl*II fragment from Ogura radish *atpA* labeled with γ-^{32}P-ATP and T_4 polynucleotide kinase; panel D, 739 bp *Spe*I-*Bal*I fragments labeled at the *Spe*I site with α-^{32}P-dATP and Klenow fragment. Nuclease mapping experiments using the 277 nt *Eco*I and *Spe*I-*Bal*I fragments from both normal and Ogura radish gave identical results; this is expected because the two sequences are identical within the transcribed region. Sizes of protection fragments and probes are shown in nt next to the corresponding panel. The migration distances of fragments in different panels are not comparable.

The 5′ end of the 2700 nt Ogura *atpA* transcript was mapped using a 700 bp end-labeled *Bgl* II fragment, extending from a *Bgl* II site approximately 350 bp 5′ to the characterized region to nucleotide – 600. When this fragment was used as a probe with sterile Ogura radish RNA, a protected fragment of 243 nt as well as low levels of fully protected probe were obtained (Fig. 4C). The 243 nt protected fragment corresponds to a 5′ transcript terminus at – 843 (Fig. 2) and predicts an Ogura *atpA* transcript of 2536 nt. This is slightly smaller than the predicted size of 2700 nt (hereafter referred to as the 2536 nt transcript) based on northern hybridization results. The presence of fully protected probe could be due to either transcription originating 5′ to the first *Bgl* II site or to the fact that a double-stranded probe was used in the nuclease mapping experiments. That a northern hybridization using a probe extending 5′ to the *Nru*I site (nucleotide – 994) failed to detect the 2536 nt transcript (Fig. 3C) suggests that – 843 is in fact the 5′ terminus of this transcript.

Several conclusions can be drawn from the transcript mapping experiments. 1) The 2536 nt Ogura *atpA* transcript results from transcription originating upstream of the 5′ rearrangement breakpoint and extending into the *atpA* coding

region. 2) A repeated sequence present in the 5′ flanking region of *atpA* and implicated in mtDNA rearrangements is transcribed elsewhere in the normal and Ogura radish mitochondrial genomes. 3) Although the 2536 nt Ogura *atpA*-associated and 900 nt repeat-associated transcripts are affected by nuclear background, neither maintain a strict correlation with the sterility/fertility phenotype when a large number of nuclear backgrounds are examined. This indicates that they are probably not causally related to Ogura CMS.

Ogura radish mitochondria contain normal levels of ATPA polypeptides

In order to determine if either the 2536 nt transcript or nuclear background affect the level of the ATPase α-subunit, antibodies were raised to the α-subunit and the size and relative amount of the polypeptide determined for several nuclear/mitochondrial combinations. An example of the results from these experiments is shown in Fig. 5. A polypeptide of approximately 55000 Da is detected in western blots of normal radish (N) and fertile (F) and sterile (S) CgGC15 radish mitochondrial proteins with the anti-α-subunit antibody. The same relative amount of α-subunit is detected in all three mitochondrial preparations. Similar levels of ATPase α-subunit were found in mitochondria isolated from all eight different nuclear backgrounds (data not shown). The lack of cross-reaction between the three mitochondrial preparations and antibody to the spinach chloroplast 32 kDa Qb (quinone-binding) protein indicates that there is little or no chloroplast contamination in the mitochondrial preparations and that all of the ATPase α-subunit cross-reactivity is due to mitochondrial protein. Likewise, equal levels of cross-reactivity are observed in all three mitochondrial preparations when western blots are treated with antibody to subunit II of the maize cytochrome oxidase (data not shown). By demonstrating that neither nuclear background nor cytoplasm affects the size or level of the α-subunit, these results support those obtained from DNA sequence analysis and transcript mapping experiments, which indicate that *atpA* is not associated with Ogura CMS.

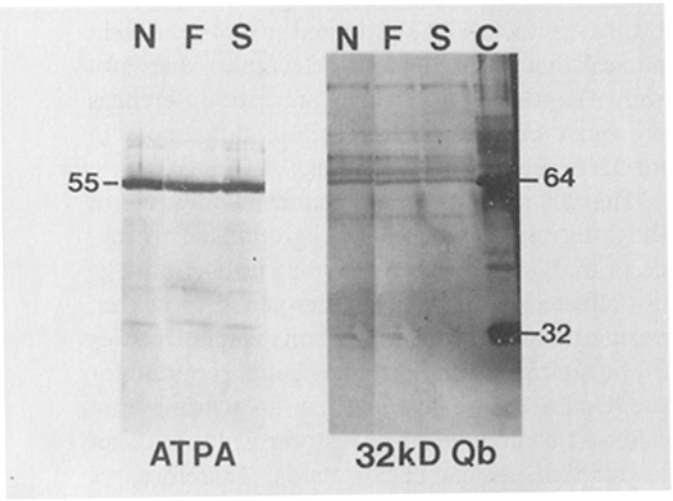

Fig. 5. Immunodetection of ATPA in normal and Ogura mitochondria. Western blots containing mitochondrial proteins (200 μg) from normal radish (N) and sterile (S) and fertile (F) CrGC15 plants as well as chloroplast proteins (C) from normal radish were treated with antisera raised against an ATPA-β-galactosidase fusion protein or the spinach chloroplast *psbA*-encoding 32 kDa protein. The 32 kDa protein dimerizes to give a 64 kDa protein band. Sizes are shown in kDa.

Discussion

We have characterized the *atpA* locus from both the normal and Ogura cytoplasms of radish to investigate rearrangements associated with *atpA* and also to determine if a 2700 nt (actual size 2536 nt) *atpA* transcript that we had previously found associated with sterile plants containing the Ogura cytoplasm could be causally related to Ogura CMS. Several lines of evidence indicate that the *atpA* locus and the 2536 nt transcript are not involved in male sterility. 1) The two loci are virtually identical (99.8%) over a 3.8 kb region that encompasses a 507 codon opening reading frame, whose product is approximately 94% identical with other plant mitochondrial ATPA polypeptides. 2) The predominant *atpA* transcript (1865 nt) is completely contained within the

unrearranged region of the locus. In addition, there are no DNA-encoded open reading frames or other structures in the Ogura *atpA* 5′ flanking region that can be implicated in male sterility. However, we can not eliminate the possibility that the RNA is edited and that the transcript contains an open reading frame not found in the DNA. 3) Analysis of *atpA* transcript patterns in several different sterile/fertile lines indicated that while the 2536 nt transcript is affected by nuclear background, it is not strictly correlated with male sterility. 4) Sterile and fertile plants possessing the Ogura cytoplasm contain an ATPA polypeptide of the same size and abundance as do normal radish plants.

The 5′ rearrangement breakpoint between the normal and Ogura *atpA* loci occurs 613 bp 5′ to the coding region and within a 689 bp repeated sequence (nucleotides −855 to −166, normal radish) that is also present in its entirety 5′ to normal radish *coxI-2*, a submolar form of the gene. This repeated sequence has been implicated in the homologous rearrangement events that generate the *coxI-2* locus (Makaroff, Apel and Palmer, unpublished). Included in this larger repeat, and immediately flanking the 5′ points of divergence between the normal and Ogura *atpA* loci as well as the normal radish *coxI-1* and *coxI-2* loci, is a 156 bp sequence that is also present 5′ to the *orf105–atp6* locus in Ogura radish [25]. Much of this region (122 bp) is homologous to a 175 bp sequence common to the 5′ flanking regions of tobacco *atp6* and *rps13* [4, 5] and *Oenothera atp6*, *coxI* and *coxIII* [15, 36]. The *Oenothera coxI-* and *coxIII*-linked copies of the repeat are part of a 657 bp sequence that has been implicated in both promoter function and mtDNA rearrangements [15]. Further work is necessary to determine what role, if any, this sequence, which is found 5′ to genes in several diverse plants and within larger repeats that have been implicated in recombination events, actually plays in plant mtRNA structure and/or function.

Transcript mapping experiments indicate that the 2536 nt *atpA* transcript found associated with sterile CrGC15 plants is the result of transcription originating 5′ to the abundant 1865 nt *atpA* transcript. The 2536 nt and 1836 nt transcripts could be related in two ways: 1) they could represent two distinct transcriptional units and nuclear background controls either transcription initiation or transcript stability or 2) the 1836 nt transcript could be a processed form of the 2536 nt transcript and nuclear background controls the level or activity of a RNA processing enzyme. Further experiments are required before we can distinguish between these possibilities. The 2536 nt transcript is not present in sterile plants containing three other nuclear backgrounds suggesting that the gene(s) that affect this transcript are not restorer genes.

The 900 nt transcript, which contains a repeat, is also affected by nuclear background (Fig. 3E). This transcript is present in fertile plants containing the Ogura cytoplasm in combination with either the CrGC15 nucleus or the 5241 nucleus. It is not present in normal radish plants, fertile Iwate plants, or in sterile Iwate, 5241, CrGC15 or *B. napus* plants containing the Ogura cytoplasm. Like the 2536 nt transcript, it is not clear at what level the nuclear-encoded factor acts. However, no differences in restriction fragment profiles of Ogura mitochondria isolated from different nuclear backgrounds are detectable (data not shown), indicating that the transcript differences are most likely not the result of differences in mtDNA genome organization.

The 900 nt transcript is found in only two of the three restored nuclear backgrounds. This suggests that, similar to the 2536 nt transcript, it is not affected by nuclear restorer genes. However, beause there is no information on the relative roles of the multiple nuclear restorer genes reported for the Ogura cytoplasm [14], it is possible that different combinations of restorer genes may act in different nuclear backgrounds. Therefore, we can not rule out the possibility that the 2536 or 900 nt transcripts may play some role in Ogura CMS that is not related to *atpA* itself.

Nuclear genes distinct from those that control fertility restoration have also been shown to affect the levels of the *urf13-T* and *orf25* transcripts in the CMS-T cytoplasm of maize [17]. In addition, nuclear effects on the translation of several mito-

chondrial proteins in sorghum [1] and TURF-13 in the CMS-T cytoplasm of maize [9] have been observed. The primary effect of the maize nuclear RF1 gene appears to be post-transcriptional, acting as an RNA processing enzyme or a transcriptional or translational factor [10, 16]. While a number of nuclear genes that are involved in yeast mitochondrial biogenesis (including DNA replication, transcription and translation factors) have been isolated and characterized [reviewed in 40], we are just beginning to characterize processes involved in plant mitochondrial biogenesis.

We have reported here the identification of two mitochondrial transcript differences in the Ogura cytoplasm of radish that appear to reflect the action of at least two nuclear loci. Further studies are necessary to determine whether these loci act at the level(s) of transcription initiation, RNA processing or RNA stability and whether they play any role in the regulation of mitochondrial function.

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