The *atp6* Coding Region Has Been Disrupted and a Novel Reading Frame Generated in the Mitochondrial Genome of Cytoplasmic Male-sterile Radish*

Christopher A. Makaroff‡, Ingrid J. Apel, and Jeffrey D. Palmer

From the Department of Biology, University of Michigan, Ann Arbor, Michigan 48109

The gene *atp6*, encoding subunit 6 of the mitochondrial F_0-ATPase complex, has been characterized from both the normal (fertile) and Ogura (male-sterile) radish cytoplasms in order to determine if previously identified *atp6* transcriptional differences could play a role in cytoplasmic male sterility. Normal radish *atp6* encodes a 262-amino acid polypeptide that exhibits approximately 80% sequence identity with other plant *atp6* polypeptides. A tRNA^{fMet} gene is located 150 base pairs 5′ to *atp6*, and the two genes may be co-transcribed. As a result of extensive rearrangement, sequences that comprise the normal *atp6* locus are present in three widely separated regions of the Ogura mitochondrial genome. Both 5′ and 3′ rearrangement breakpoints have been identified and found to be associated with short repeated sequences. The normal and Ogura *atp6* loci share a common 987-base pair region containing most of the *atp6* coding region and 106 base pairs of the 3′-flanking region. A 105-codon open reading frame is transcribed as the first gene of an Ogura *atp6* bicistronic mRNA. This sequence is not present in normal radish mitochondrial DNA. There are many nucleotide differences in the Ogura *atp6* 5′-flanking and coding regions that can be expected to eliminate normal translation of *atp6*. At least two possibilities exist for translation of Ogura *atp6*, both of which would result in an NH_2-terminal amino acid sequence different from that of normal radish.

Cytoplasmic male sterility (CMS)[1] is a maternally inherited trait in which plants fail to produce functional pollen but maintain female fertility (1–3). The CMS trait is associated with mitochondrial dysfunction. Alterations in mitochondrial DNA (mtDNA) structure have been identified in every CMS plant examined to date (for a review, see Ref. 3). In addition, CMS-specific transcription and/or translation patterns have been found in maize (4–8), sorghum (9, 10), petunia (11), sunflower (12), and radish (13). Nuclear genes have been identified that restore fertility to CMS cytoplasms (2, 3).

The original observation of CMS in the crucifers (family Cruciferae) was by Ogura (14) who found male-sterile radish (*Raphanus sativus*) plants growing among an escaped radish cultivar in Japan. In Ogura radish, meiosis proceeds normally up to early microspore formation when a collapse of the tapetal tissue leads to microspore degeneration (14, 15). Three nuclear loci have been identified in European cultivars of radish that restore fertility to the Ogura cytoplasm (15, 16).

Ogura CMS, like all other examples of CMS, is found to be associated with mitochondrial alterations. We have recently shown that the Ogura mitochondrial genome is highly rearranged relative to that of normal (*i.e.* male-fertile) radish (13). In addition, altered transcript patterns were identified for three mitochondrial genes: *atpA*, *atp6*, and *coxI*. The transcript pattern for one of these, *atpA*, was found to be affected by nuclear restorer genes. In order to identify the alteration(s) responsible for Ogura CMS, we have further characterized the Ogura mitochondrial genome and report here a detailed analysis of the *atp6* locus from both normal and Ogura radish. An Ogura-specific *atp6* transcriptional unit containing both a disrupted *atp6* coding region and a novel 105-codon ORF has been generated as a result of multiple rearrangements. Numerous nucleotide differences at the 5′ end of Ogura *atp6* eliminate the normal radish translation start site. The possible roles of these alterations in Ogura CMS are discussed.

MATERIALS AND METHODS

Plant Material—The *R. sativus* cultivar Scarlet Knight was the source of the normal (wild-type) cytoplasm. The mitochondrial genome of Scarlet Knight exhibits the same restriction enzyme profiles as those of three other commonly used male-fertile radish cultivars (17)[2] 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 source of the Ogura cytoplasm. Sterile and fertile (nuclear restored) plants containing the Ogura cytoplasm were distinguished by their ability to produce functional pollen.

Isolation of Nucleic Acids—Mitochondrial DNA was isolated from leaves of 6-week-old plants by the DNase I procedure (18). Mitochondrial RNA (mtRNA) was isolated in the presence of aurintricarboxylic acid from mitochondria purified by differential centrifugation (19). Plasmid DNA was isolated using the alkaline lysis procedure (20).

Sequence Determination and Analysis—Normal and Ogura *atp6* were isolated previously on 3.1- and 19.4-kb SalI clones in pUC8 and pTZ18, respectively (13, 21). Standard techniques were used for the preparation of recombinant plasmids in *Escherichia coli* (22). Exonuclease III deletion clones (23) were generated from plasmids containing either a 2.4-kb BamHI fragment in pTZ19R (Ogura *atp6*) or a 3.1-kb SalI fragment in pTZ18R (normal *atp6*) prior to sequencing by the dideoxy chain termination procedure of Sanger (24). The normal radish *atp6* 5′-flanking sequence was localized to a 0.49-kb XbaI/ClaI fragment from the Ogura 5.1-kb SalI fragment (13) by Southern analysis. This fragment was then cloned into Bluescript

[1] The abbreviations used are: CMS, cytoplasmic male sterility; bp, base pair(s); kb, kilobase pair(s); PIPES, piperazine-*N,N*′-bis[2-ethanesulfonic acid]; ORF, open reading frame.

[2] C. A. Makaroff and J. D. Palmer, unpublished data.

SK$^+$ and SK$^-$ and sequenced. DNA sequences were analyzed on a Sun Microsystems, Inc. minicomputer using the Eugene program (Department of Cell Biology, Baylor College of Medicine, Houston, TX).

Southern Analysis of atp6 and Flanking Regions—In order to identify the position and orientation of specific rearranged fragments, exonuclease III-generated subclones containing various segments of the normal and Ogura *atp6* loci were hybridized to Southern blots containing a series of single and double restriction digests (*Pst*I, *Sal*I, *Bgl*I, and *Nru*I) of Ogura and normal radish mtDNAs (see Ref. 13 for restriction fragment profiles and maps of the two genomes). Methods used for restriction endonuclease digestion, agarose gel electrophoresis of DNA fragments, bidirectional transfer from agarose gels to Zetabind (AMF Cuno) nylon membranes, and labeling of recombinant DNA fragments by nick translation were as described previously (22, 25).

Northern Hybridization and Nuclease Mapping—Conditions for the electrophoresis, transfer, and hybridization of RNA (7.5 μg/lane) to nick-translated probes were described previously (13). Nuclease-mapping experiments were conducted essentially as described (26, 27). RNA (10 μg) was ethanol-precipitated together with a DNA probe that was labeled with either [α-^{32}P]dATP by primer extension of single-stranded DNA using the Klenow fragment of DNA polymerase I (28) or [γ-^{32}P]ATP with polynucleotide kinase (22). The precipitate was 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 6–16 h at 46–56 °C (the GC content of the probe determined the annealing temperature). Samples were treated with 200 units of mung bean nuclease in 200 μl of a buffer containing 50 mM NaCl, 30 mM sodium acetate, pH 5.2, and 1 mM EDTA for 60 min at 37 °C. The samples were then phenol-extracted, ethanol-precipitated, resuspended in denaturing stop dye, and electrophoresed on 5% nondenaturing polyacrylamide gels next to ^{32}P-labeled Bluescript KS$^+$ digested with *Hin*fI. Samples were also run on high resolution 6% polyacrylamide denaturing gels (29) next to dideoxy sequencing ladders for precise size determinations.

RESULTS

Normal Radish atp6—The nucleotide sequence of a 3.1-kb *Sal*I fragment from normal radish was determined by dideoxy sequencing of deletion clones generated by exonuclease III (Fig. 1) and is shown in Fig. 2. This fragment contains a 262-codon open reading frame capable of encoding a 29,130-Da polypeptide that is identified as *atp6* by DNA sequence homology. The deduced amino acid sequence exhibits 80–82% identity with *atp6* polypeptides from maize (30), *Oenothera* (31), and tobacco (32). The AUG codon (nucleotides 1–3) assigned as the putative initiator methionine is the only in-frame methionine that precedes the highly conserved *atp6*-homologous sequence block (Figs. 2 and 3). Located 12 bp 5′ to 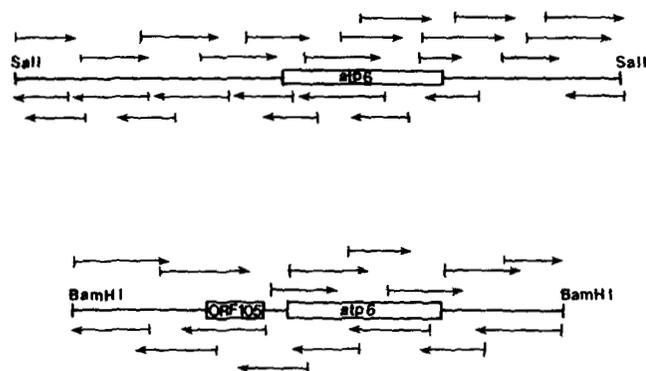the putative initiator methionine is a 9-bp sequence that is 100% complementary to the 3′ end of plant 18 S rRNAs and probably serves as a ribosome binding site (40). The NH$_2$-terminal sequence of radish *atp6* is more similar in length to that observed in fungal rather than plant mitochondrial *atp6* genes (Fig. 3). Fungal *atp6* genes encode short (5–10 amino acids) presequences that are proteolytically cleaved to yield a mature protein with an NH$_2$-terminal serine residue (39). In normal radish *atp6*, nine amino acids precede the highly conserved serine (Fig. 3), which has been shown to be the NH$_2$-terminal residue of the mature *Saccharomyces cerevisiae* (38) and wheat polypeptides (39). Other plant *atp6* presequences could range in length from 4–144 amino acids (Fig. 3), but the actual translation initiation site is not certain.

Located on the same strand 150 bp 5′ to normal *atp6* is a gene for tRNA^{fMet} (*trnfM*). This radish sequence exhibits the typical cloverleaf structure and is 88 and 96% identical to bean tRNA^{fMet} (41) and maize *trnfM* (42), respectively. The 3′-terminal nucleotides CCA-OH characteristic of all mitochondrial tRNAs are not encoded in this gene, as has been observed in maize tRNA genes (42).

Nuclease mapping experiments using a uniformly labeled probe extending from within the normal *atp6* coding sequence (nucleotide 55) to the *Sal*I site at its 5′ end produced protected fragments of 196, 207, and 1347 bp (Fig. 4). These protected fragments correspond to two major 5′ transcript termini at nucleotides −152 and −141 (Fig. 2) and the fully protected probe. The transcript termini at −152 and −141 are 9 and 20 bases from the 3′ end of the *trnfM* gene, respectively, suggesting that the transcripts may represent processed forms of a larger transcript and that the two genes may be co-transcribed. This hypothesis is supported by the presence of a 2300-nucleotide *atp6* transcript in Northern hybridization experiments (Fig. 5), which is also identified with the *trnfM*-containing 0.49-kb *Xba*I/*Cla*I fragment from Ogura radish (data not shown) and the presence of low levels of fully protected probe in nuclease mapping experiments. Analysis of the DNA sequences immediately 5′ and 3′ to *trnfM* failed to identify sequences thought to be involved in transcript processing in plant mitochondria (43). Perhaps the tRNA cloverleaf structure itself acts as a processing signal, as has been postulated for the RNA processing events in animal mitochondria (44).

Located 97 bp 3′ to normal radish *atp6* is a 249-bp duplication of part of the radish *coxI* locus, containing 117 bp of 5′-flanking sequence and the first 44 codons of the *coxI* coding region.[3] The *coxI* sequences are 99.6% identical.

Ogura Radish atp6—Ogura radish *atp6* was subcloned from a previously characterized 19.4-kb *Sal*I clone (13) and sequenced as a 2.5-kb *Bam*HI fragment (Fig. 1). This 2.5-kb Ogura fragment and the 3.1-kb *atp6*-containing fragment from normal radish share a common 987-bp region. Most of the *atp6* coding region (754 bp) and part of the 3′-flanking sequence (106 bp) are highly conserved (99.7% identical), whereas a 118-bp segment containing the 5′-coding region and its flanking sequence exhibits only 75% sequence identity (Fig. 2). Upstream of nucleotide −91 and downstream of nucleotide 887, the sequences are unrelated with the exception of 66 bp (region A; positions −399 to −334 in normal radish and −764 to −699 in Ogura radish) common to both sequences.

There are 13 nucleotide differences between the Ogura and normal *atp6* coding regions, all within the first 13 codons. The most striking changes are the A → C (nucleotide 1) and G → T (nucleotide 3) that change the putative initiator methionine to a leucine codon (Figs. 2 and 3). There are 33

FIG. 1. **Sequencing strategy for the *atp6* locus from normal (*top*) and Ogura (*bottom*) radish.** *Open boxes* represent the ORF-105 and *atp6* open reading frames. *Arrows* indicate the length and orientation of each sequence determination. All clones were generated by the exonuclease III procedure.

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[3] C. A. Makaroff, I. J. Apel, and J. D. Palmer, unpublished data.

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Left column sequences:

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GTCGACGAGAACGGGAAAGGGCGCAGCTCCGACAGTTTTTCTCTTAAGTTGGAATCCCAT
-1264                                                  -1234

AGATGAATAAAGTCAGGGAATCTACATGTGTTAAGCACCATTTAAGAGAAGCTAGCGTCC
-1204                                                  -1174

GATTCCATGCTTCCCTATGCTATAGAATAGAATGACGATGTCTTTGCCTGTCTAGTTAAT
-1144                                                  -1114

               GGATCCCAAAGATAGGCATTGTTCGCCTTGGCCTTGG
AAATCTCTTTCGTTGGAGAAGGAGCTGGCATCTTATCCGCGTTGCTAATATTTGGGAGGT
-1084                                                  -1054

ACCTCAAGGAGGAATAGATAACCTAAGTCTTATCTTATAGGGGGCCTCGCAATCATAATC
TAATCAATAGGGGAATCAGCTGGTATTTCATCTTCCTCTATAATTTTGTGATTTCACGGA
-1024                                                   -994

AGTTGAGGGGAGGGAATGAAATGAATCTGAAGCAGCAGATAGCCACACCACAGGATAATC
CATCATTCATTAAACCAAACAAGGACCAGTCGAAGGAGTAAATTACCTCTTTGTACATAT
-964                                                    -944

CATTCCTACCTTAGAAAAGGGAAGGCCGTGAGGGTAAGGTAAAGTTGAATACTACTAAAAA
ACTATACTCATACCCCTATTACGATACAGATTCACCCTATCTTGCTTTCACGGTGAACGA
-904                                                    -874

AGCTGGTGAAAGACCTTGGTGAAAATCCTCGGGTATCGACGGAATCCCATTCGTTTGAAA
CCTGCTTTGACCGGGAACCAAAGAGCCAATACACCAACAACCAAGGAACGGGAGTGGGTG
-844                                                    -814

CTAATACTTATCCCCCCCTTCCAAATTGATCATATCTGACTATTGAGGAGAGCTTATCGA
GTCCCTAGGAACGCCCTTCTTTGAGTAAGTCCTTGGTCTAAGTGCTGCCCTATCTAGTTA
                               -784                     -754
            A
TTGACAGAATAATACGTATATAAGAAGAAGGCCGCTTTGTGGAGTGAGCTTTCTCTCTAA
TCCCGGTATCGGTCTTTCTTTAGTCTCTGGCGTTCAGACGCTAGTTATTGAGCTCCCCTG
-724                                                    -694

CCTACAACTCTTACTAAACTAAACAAAGTTCAATCTCATAAGAGAAGAAATCTCTATGCC
GTCTGAGAAGGTAGCTCTCCTAGTAGCAGTTGTCATTGCTACTCTACTATGGACCAACCT
-664                                                    -634
            B
CCCTGTTCTTGGTTTTCTCCCATGCTTTTGTTGGTCAACAACCAACCACAACTTTCTATA
ATCACCTATCCTTGTACATAACTGCATTTGTAATAGAATAGGTTCTTCTTTCTCTGTGCT
-604                                                    -574

GTTCTTCACTACTCCTAGAGGCTTTCCTAATGAAGCTGTCTGGAGGGATTTGTTGAAATC
ATATCCCAAAGCGCTTTCATTCGTAGCATCAATCAAGTCAGTTTATGAAGGGAAATCCGTT
-544                                                    -514
     ORF105
           M  R  R  I  F  Q  F  D  E  T  S  L  N  S  S  S
AATTAATCCAAAATGCGACGAATCTTTCAATTTGATGAAACTAGCCTTAATTCAAGTTCC
GAACAAATCACTCCGTGCCTCCCCTCAAGCACGACAGATTGACTTTGATAAGCGCACTAG
-484                                                    -454
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Right column sequences:

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17              D                                        A
 S  D  T  Y  S  R  T  A  S  Q  S  T  T  T  I  N  D  Y  S  L
AGTGATACATATTCTAGAACTGCGAGTCAATCCACGACGACTATTAACGATTATAGTTTG
CTGACTACGAGATCAGTCTCACCTCGAGTTCGACTACGGAACGGAACCCCACCCGTGCTT
                 -424                                   -394
37              D
 K  S  S  S  S  D  P  Y  S  R  T  A  S  Q  S  T  N  F  S  S
AAATCAAGTTCCAGTGATCCATATTCTAGAACTGCGAGTCAATCCACTAACTTTTCTAGT
ATCGATTGATAGAATAATACGTATATAAGAAGAAGGCTGCTTTGTGGAGTGATCTTTCTC
                 -364                                   -334
57              E                          E
 G  Q  R  P  E  Y  R  M  L  M  L  V  V  K  R  P  V  L  R  M
GGTCAACGTCCGGAATACAGGATGCTTATGCTAGTGGTCAAGCGTCCGGTTCTCAGGATG
GAAATGAATTAAGTAAGGGCGCTATGTTCAGATTCTGAACCAAAGCACTAGTTGAGGTCT
                 -304                                   -274
77         L  M  L  V  V  K  R  P  V  L  S  M  L  M  L  V  G  F  L  K
CTTATGCTAGTGGTCAAGCGTCCGGTACTCAGCATGCTTATGCTAGTGGGATTTTTGAAG
GAAAGCCTTATGAGCAGAAGTAATAAATACCTCGGGGAAGAGCCCGGTAGAGGAATTGG
                 -244      trnfM                        -214
97
 N  Q  S  R  S  E  S  Y  R
AATCAATCCAGGTCTGAATCCTACCGATGAGCGTGTAGTAGAGCTGCAATGTATGATACG
TCAACTCATCAGGCTCATGACCTGAAGATTACAGGTTCGAATCCTGTCCCCGCAGTAAGT
                 -184                                   -154
CGAGAATTTTGAGGTATTCAACGTGCGATCCTCAAAGGGCCTTGAATCTCGCAGAGGCTT
AAGGGTTTCATTCTGCATCACTCTCCCCGTCGTTCTCGACCTCGCAAGGTTTTTGAAGCG
                 -124                                   -94
CA - T GA A   CA    T C       T GG T C     A               C C
GCCGAAGCGGGAAGTGACAATACCGCTTTTCTTCAGCACATTTTGGATGATTTGAGCGAA
                 -64                                    -34
                                                        27
          TCCG GAATC T   CGA  A  C TG     GG A A C     GCC
AACGGAGTACAAAGTTCAGCCTTTAAGGAGGCTATGAATCAAATAGGGCTGCTGGCGCAG
                 -4     M  N  Q  I  G  L  V  A  Q
                        atp6
                           AG              57              87
TCCCCACTTGACCAATTTGAGATTGTCCCATTGATTCCTATGAATATCGGAAACTTCTAT
 S  P  L  D  Q  F  E  I  V  P  L  I  P  M  N  I  G  N  F  Y
10
                           117             147
TTCTCATTCACAAATCCATCTTTGTTCATGCTGCTAACTCTGAGTTTTTTCCTACTTCTG
 F  S  F  T  N  P  S  L  F  M  L  L  T  L  S  F  F  L  L  L
30
                           177             207
ATTCATTTTATTACTAAAAAGGGAGGAGGAAACTTAGTCCCAAATGCTTGGCAATCCTTG
 I  H  F  I  T  K  K  G  G  G  N  L  V  P  N  A  W  Q  S  L
50
                           237             267
GTAGAGCTTCTTTATGATTTCGTGCTGAACCTGGTAAAGGAACAAATAGGTGGTCTTTCC
 V  E  L  L  Y  D  F  V  L  N  L  V  K  E  Q  I  G  G  L  S
70
```
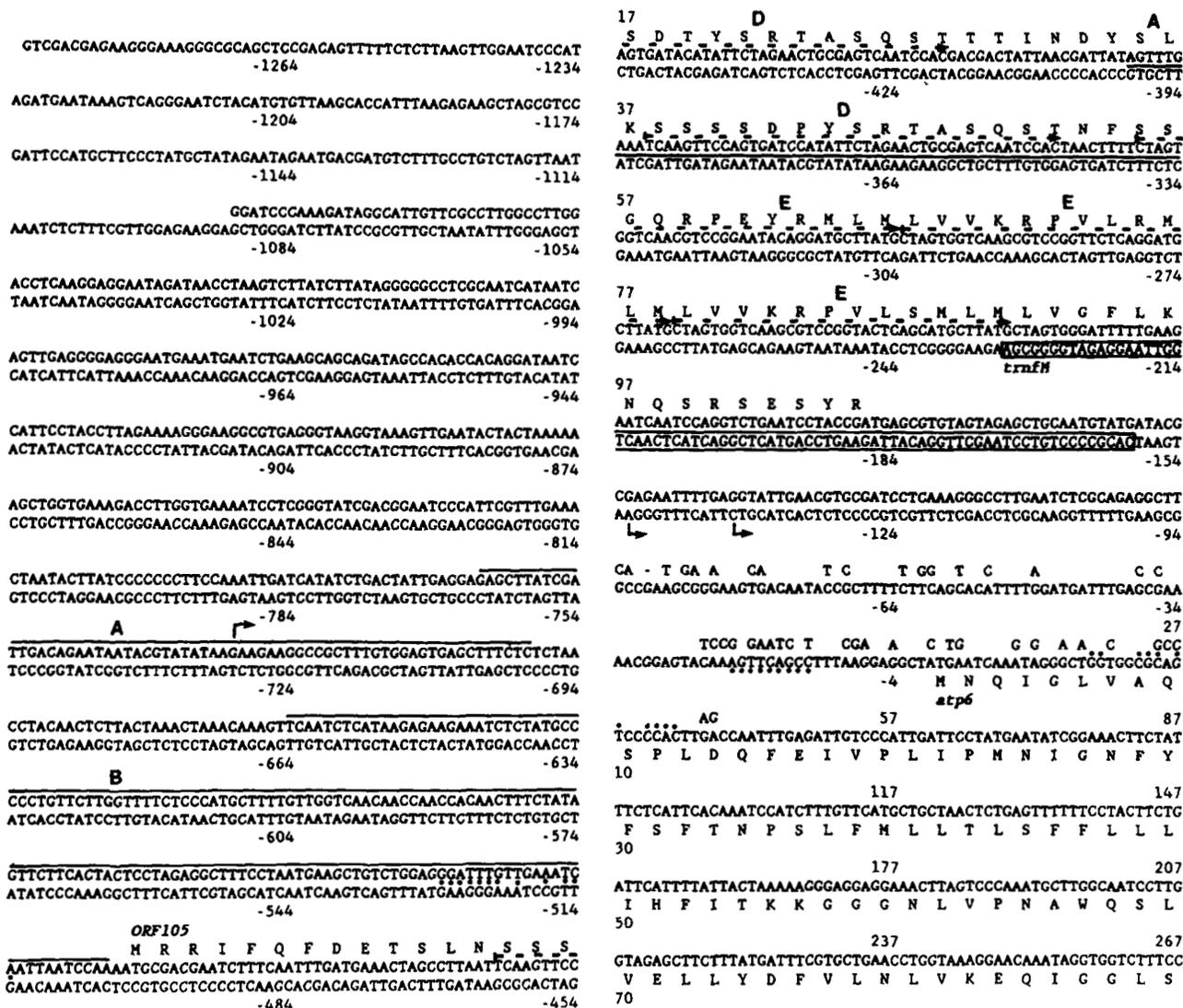
FIG. 2. Nucleotide and derived amino acid sequences of the *atp6* loci from normal and Ogura radish. The Ogura sequence (*top line*) is aligned with that from normal radish (*bottom line*). Where the two are identical, only the normal sequence is shown. Numbering of nucleotides is from the predicted translational start of *atp6* in normal radish. Amino acids are shown *above* (ORF-105) and *below* (*atp6*) the nucleotide sequence and are numbered relative to the predicted translation initiation codons. A *trnfM* gene present upstream of *atp6* in normal radish is *boxed*. Nucleotides complementary to the last 17 nucleotides of the 18 S rRNA, allowing for G-U base pairing, are indicated with *solid circles* under the corresponding sequence; where the two sequences are identical, the *circles* appear above the normal sequence. The 66-bp repeat (*A*), the *atpA*- and *coxI*-homologous sequence (*B*), and the *coxI*-homologous sequence (*C*) are *overlined* (*solid line*); the 44-bp (*D*) and 35-bp (*E*) direct repeats within ORF-105 are *overlined* with *dashed lines*. Major 5′ transcript termini are represented by *arrows* (below the sequence for normal radish and above the sequence for Ogura radish).

additional differences in the 91 bp immediately upstream of the coding region. Seven of these changes occur in the proposed ribosome binding site of normal *atp6*. Together these changes can be expected to eliminate normal translation of Ogura *atp6*. The leucine codon in Ogura radish that corresponds to the initiator methionine in normal radish is preceded by a 44-codon open reading frame; however, there are no in-frame methionine codons between the leucine codon and the first in-frame stop codon (Figs. 2 and 3). Located 23 amino acids into the coding region is a second methionine that is preceded by a sequence which shows limited sequence identity with the proposed plant mitochondrial ribosome binding site (40).

Ogura atp6 Is Co-transcribed with a Novel 105-Amino Acid ORF—Upstream of Ogura *atp6* is a 105-codon open reading frame (ORF-105) that is capable of encoding a 12,052-Da

polypeptide. This open reading frame may have been generated through a series of duplication and rearrangement events. Regions of 44 and 35 bp that are directly repeated a total of two and three times, respectively, are found within the ORF-105 coding region. Northern analysis indicates that ORF-105 is co-transcribed with *atp6* in Ogura mitochondria (Fig. 5). An ORF-105-homologous transcript is not detected in mtRNA from normal radish, consistent with the absence of this sequence from normal radish mtDNA (see below). Hybridization of the Ogura clones to multiple transcripts in both sterile and fertile Ogura mtRNAs (Fig. 5) is most likely due to the presence of repeated sequences on the Ogura clones. When normal radish or *Brossica campestris atp6* clones are used to probe these RNA preparations, several low abundance transcripts are identified, but the 1400-nucleotide transcript is the only one of the abundant transcripts shown in Fig. 5 that is
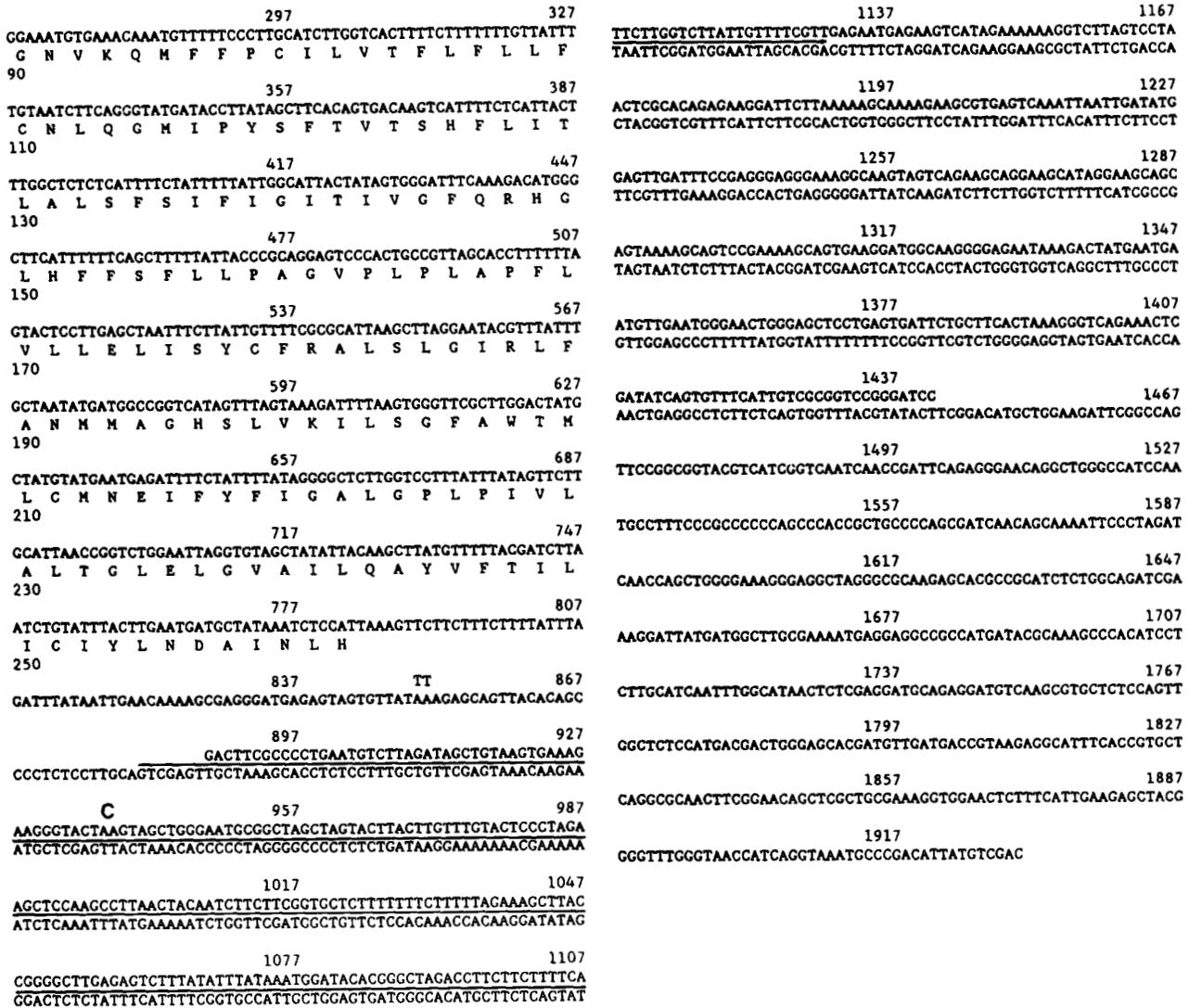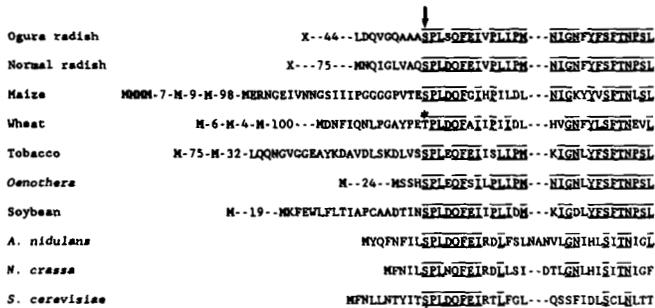
Fig. 2 (continued) — left column

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        297                              327
GGAAATGTGAAACAAATGTTTTTCCCTTGCATCTTGGTCACTTTTCTTTTTTTGTTATTT
 G  N  V  K  Q  M  F  F  P  C  I  L  V  T  F  L  F  L  L  F
90

        357                              387
TGTAATCTTCAGGGTATGATACCTTATAGCTTCACAGTGACAAGTCATTTTCTCATTACT
 C  N  L  Q  G  M  I  P  Y  S  F  T  V  T  S  H  F  L  I  T
110

        417                              447
TTGGCTCTCTCATTTTCTATTTTTATTGGCATTACTATAGTGGGATTTCAAAGACATGGG
 L  A  L  S  F  S  I  F  I  G  I  T  I  V  G  F  Q  R  H  G
130

        477                              507
CTTCATTTTTTCAGCTTTTTATTACCCGCAGGAGTCCCACTGCCGTTAGCACCTTTTTTA
 L  H  F  F  S  F  L  L  P  A  G  V  P  L  A  P  F  L
150

        537                              567
GTACTCCTTGAGCTAAATTTCTTATTGTTTTCGCGCATTAAGCTTAGGAATACGTTTATTT
 V  L  L  E  L  I  S  Y  C  F  R  A  L  S  L  G  I  R  L  F
170

        597                              627
GCTAAATATGATGGCCGGTCATAGTTTAGTAAAGATTTTAAGTGGGTTCGCTTGGACTATG
 A  N  M  M  A  G  H  S  L  V  K  I  L  S  G  F  A  W  T  M
190

        657                              687
CTATGTATGAATGAGATTTTCTATTTTATAGGGGCTCTTGGTCCTTTATTTATAGTTCTT
 L  C  M  N  E  I  F  Y  F  I  G  A  L  G  P  L  P  I  V  L
210

        717                              747
GCATTAACCGGTCTGGAATTAGGTGTAGCTATATTACAAGCTTATGTTTTTACGATCTTA
 A  L  T  G  L  E  L  G  V  A  I  L  Q  A  Y  V  F  T  I  L
230

        777                              807
ATCTGTATTTACTTGAATGATGCTATAAATCTCCATTAAAGTTCTTCTTTCTTTTATTTA
 I  C  I  Y  L  N  D  A  I  N  L  H
250

        837              TT           867
GATTTATAATTGAACAAAAGCGAGGGATGAGAGTAGTGTTATAAAGAGCAGTTACACAGC

        897                              927
                GACTTCGCCCCTGAATGTCTTAGATAGCTGTAAGTGAAAG
CCCTCTCCTTGCAGTCGAGTTGCTAAAGCACCTCTCCTTTGCTGTTCGAGTAAACAAGAA
        C
        957                              987
AAGGGTACTAAGTAGCTGGGAATGCGGCTAGCTAGTACTTACTTGTTTGTACTCCCTAGA
ATGCTCGAGTTACTAAACACCCCCTAGGGGCCCCCTCTCTGATAAGGAAAAAAACGAAAAA

        1017                             1047
AGCTCCAAGCCTTAACTACAATCTTCTTCGGTGCTCTTTTTTTCTTTTTACAAAGCTTAC
ATCTCAAATTTATGAAAAATCTGGTTCGATCGCTGTTCTCCACAAACCACAAGGATATAG

        1077                             1107
CGGGGGCTTGAGAGTCTTTATATTTATAAATGGATACACGGGCTAGACCTTCTTCTTTTCA
GGACTCTCTATTTCATTTTCGGTGCCATTGCTGGAGTGATGGGCACATGCTTCTCAGTAT
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Fig. 2 (continued) — right column

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        1137                             1167
TTCTTGGTCTTATTGTTTTCGTTGAGAATGACGAAGTCATAGAAAAAAGGTCTTAGTCCTA
TAATTCGGATGGAATTAGCACGACGTTTTCTAGGATCAGAAGGAAGCGCTATTCTGACCA

        1197                             1227
ACTCGCACAGAGAAGGATTCTTAAAAAGCAAAAGAAGCGTGAGTCAAATTAATTGATATG
CTACGGTCGTTTCATTCTTCGCACTGGTCGGGCTTCCTATTTGGATTTCACATTTCTTCCT

        1257                             1287
GAGTTGATTTCCGAGGGAGGGAAAGGCAAGTAGTCAGAAGCAGGAAGCATAGGAAGCAGC
TTCGTTTGAAAGGACCACTGAGGGGGATTATCAAGATCTTCTTGGTCTTTTTCATCGCCG

        1317                             1347
AGTAAAAGCAGTCCGAAAAGCAGTGAAGGATGGCAAGGGGAGAATAAAGACTATGAATGA
TAGTAATCTCTTTACTACGGATCGAAGTCATCCACCTACTGGGTGGTCAGGCTTTGCCCT

        1377                             1407
ATGTTGAATGGGAACTGGGAGCTCCTGAGTGATTCTGCTTCACTAAAGGGTCAGAAACTC
GTTGGAGCCCTTTTTATGGTATTTTTTTTCCGGTTCGTCTGGGGAGGTAGTGAATCACCA

        1437                             1467
GATATCAGTGTTTCATTGTCGCGGTCCGGGATCC
AACTGAGGCCTCTTCTCAGTGGTTTACGTATACTTCGGACATGCTGGAAGATTCGGCCAG

        1497                             1527
TTCCGGCGGTACGTCATCGGTCAATCAACCGATTCAGAGGGAACAGGCTGGGCCATCCAA

        1557                             1587
TGCCTTTCCCGCCCCCCAGCCCACCGCTGCCCCAGCGATCAACAGCAAAATTCCCTAGAT

        1617                             1647
CAACCAGCTGGGGAAAGGGAGGCTAGGGCGCAAGAGCACGCCCGCATCTCTGGCAGATCGA

        1677                             1707
AAGGATTATGATGGCTTGCCGAAAATGAGGAGGCCGCCCATGATACGCAAAGCCCACATCCT

        1737                             1767
CTTGCATCAATTTGGCATAACTCTCGACGGATGCAGAGGATGTCAAGCGTGCTCTCCAGTT

        1797                             1827
GGCTCTCCATGACGACTGGGAGCACGATGTTGATGACCGTAAGAGGCATTTCACCGTGCT

        1857                             1887
CAGGCGCAACTTCGGAACAGCTCGCTGCCGAAAGGTGGAACTCTTTCATTGAAGAGCTACG

        1917
GGGTTTGGGTAACCATCAGGTAAATGCCCGACATTATGTCGAC
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Fig. 2—*continued*

FIG. 3. Alignment of the deduced NH₂-terminal amino acid sequence of normal and Ogura radish *atp6* with those from maize (30), tobacco (32), *Oenothera* (31), soybean (33), wheat (34), *Aspergillus nidulans* (35), *Neurospora crassa* (36), and *S. cerevisiae* (37). Identical amino acids are *overlined* and *underlined*, and inframe methionines are shown in **boldface**. In-frame stop codons in the radish sequences are represented by an X. The position of the NH₂-terminal serine residue of the mature proteins as determined for wheat (38) and *S. cerevisiae* (39) is indicated with an *arrow*. (*, a discrepancy exists between the deduced amino acid sequence of wheat *atp6* and the NH₂-terminal protein sequence data; see Footnote 4.)

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detected (data not shown; Ref. 13). The large, low abundance transcripts may be unprocessed forms of the *atp6* transcript (see below).

Nuclease mapping experiments using a probe end-labeled at the *Xba*I site (nucleotide −437) and extending to the *Bam*HI site (nucleotide −1090) resulted in protected fragments of 293 and 653 nucleotides (Fig. 4). These protected fragments correspond to a 5′ transcript terminus centered at −730 and the fully protected probe. This site (−730) is located within repeated region A (Fig. 2). This result, along with that from Northern analysis, indicates that the 5′ transcript terminus at −730 is most likely a processed form of a primary transcript that extends upstream of the sequenced region. Nuclease mapping experiments using probes labeled at a site within the *atp6* coding region failed to identify any shorter Ogura *atp6* transcripts (data not shown). This result is also consistent with Northern experiments that indicate that ORF-105 and *atp6* are transcribed together as part of a polycistronic RNA in Ogura radish.

Although we do not yet know if ORF-105 is translated, its codon usage is similar to that of the normal and Ogura radish *atp6*, *atpA*, and *coxI* genes.[3] In addition, it is preceded by a 16-bp sequence that is 75% complementary to the 3′ end of plant 18 S rRNAs (40). Because there is no similarity between
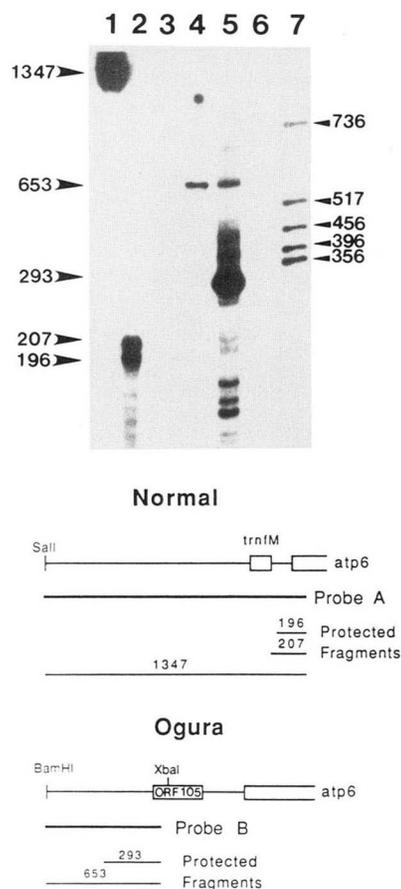
FIG. 4. Nuclease mapping of the 5′ end of normal and Ogura radish *atp6* transcripts. *Top*, autoradiographs of nuclease mapping of normal (1–3) and Ogura (4–6) *atp6* using the probes A and B, respectively (shown below). *Lane 1*, probe A; *lane 2*, probe A plus 10 µg of normal radish mtRNA treated with mung bean nuclease; *lane 3*, probe A plus 20 µg of yeast tRNA treated with mung bean nuclease; *lane 4*, probe B; *lane 5*, probe B plus 10 µg of Ogura radish mtRNA treated with mung bean nuclease; *lane 6*, probe B plus 20 µg of yeast tRNA treated with nuclease; *lane 7*, ³²P-labeled Bluescript KS⁺ digested with *Hin*FI. Protected fragments are marked with *arrows*. Sizes are shown in base pairs. *Bottom*, schematic illustration of probes used in nuclease mapping and the resulting protected fragments. Probe A (*heavy line*) was a 1347-bp fragment extending from −55 to the beginning of the clone, nucleotide −1292, and was uniformly labeled as the complement of the *atp6* mRNA by primer extension. Protected fragments (*thin lines*) of 196, 207, and 1347 nucleotides were observed with probe A. Probe B (*heavy line*) was a 653-bp fragment extending from −437 to −1089 that was end-labeled with polynucleotide kinase. Protected fragments (*thin lines*) of 293 and 653 nucleotides were observed with probe B.

FIG. 5. Transcriptional patterns of *atp6* and ORF-105 from Ogura radish. *Top*, transcript patterns observed when mitochondrial RNAs (7.5 µg) from normal radish (*N*), sterile Ogura (*S*), and nuclear-restored Ogura radish (*F*) were probed with clones A and B, whose locations are shown below. *Bottom*, schematic representation of the Ogura *atp6* locus and the probes used in the Northern analysis.
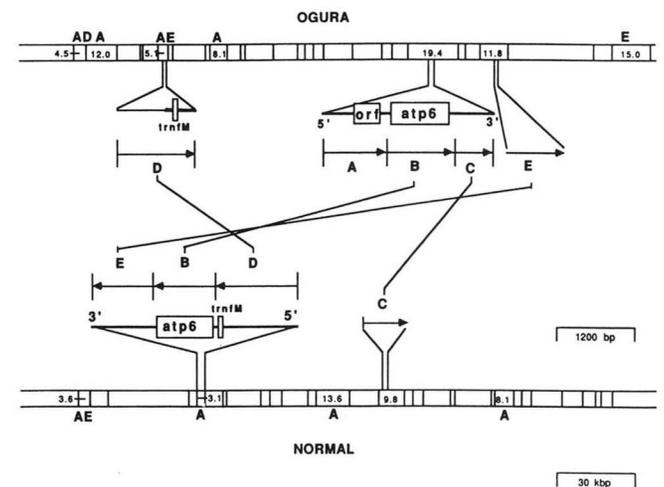
FIG. 6. *Sal*I restriction maps of the master chromosome and schematic representations of the *atp6* locus in the genomes of Ogura (*top*) and normal (*bottom*) radish. *Heavy lines* represent sequenced regions and correspond to the upper size scale. *Arrows* represent the position and relative orientation of homologous sequence blocks, as determined by Southern hybridization using region-specific probes labeled *A–E*. *Crossing lines* connect homologous sequence blocks. *Block A* is not present in normal radish. *Letters* above and below the Ogura and normal radish genomic maps, respectively, indicate additional fragments that exhibit weak cross-hybridization with the indicated regions.

ORF-105 and any sequences in the data banks, the function of the putative polypeptide is unknown.

Genomic Organization of the atp6 Loci—To determine which normal radish sequences comprise the Ogura *atp6* locus and where on the Ogura mitochondrial genome the normal *atp6* flanking sequences reside, a series of Southern hybridizations was conducted. Deletion clones containing the *atp6* coding region, as well as the normal and Ogura radish 5′ and 3′-flanking regions, were used to probe Southern blots containing single and double digests of normal and Ogura radish mtDNA. A summary of these results is shown in Fig. 6. The Ogura *atp6* locus can be divided into three regions (A, B, and C), two of which are present in normal radish mtDNA (B and C). Region B, the *atp6* coding region, is separated by 77 kb from region C, the Ogura *atp6* 3′-flanking sequence, in normal radish mtDNA. Most of region A, which contains the Ogura

atp6 5′-flanking region and ORF-105 is not present in normal radish mtDNA. However, a probe from this region hybridizes weakly to many fragments in both DNAs, indicating that there are sequences present in this segment that are repeated at least four times in each genome (Fig. 6). Consistent with this result is the presence of a 162-bp repeated sequence, nucleotides −664 to −503 (Fig. 2), that has been identified upstream of normal radish *atpA* and *coxI* and Ogura *coxI* by sequence analysis.[3] Thus, much of region A may be present in normal radish but in an extensively rearranged form. The three segments that comprise the normal radish *atp6* locus are widely separated in the Ogura radish mitochondrial genome (Fig. 6). Part of the normal radish 3′ flank (region E),

which contains the *coxI*-homologous sequence, is also repeated in the two genomes.

Rearrangement Breakpoints—The normal radish *atp6* 5′-flanking region (region D) was used in Southern hybridization experiments to identify an *XbaI/ClaI* 0.49-kb clone, which contains this region in Ogura radish mtDNA. This fragment was sequenced and aligned with the normal and Ogura *atp6* 5′ flanks to identify the 5′ rearrangement breakpoint (Fig. 7). The *XbaI/ClaI* 0.49-kb fragment shares a 66-bp sequence present in the 5′-flanking region of both normal and Ogura *atp6*. In addition, this fragment contains the *trnfM* gene that is present upstream of normal radish *atp6* and co-transcribed with it. Because there is no sharp break in sequence identity, the exact site of rearrangement cannot be determined. The number of nucleotide differences (75% sequence identity) in a 118-bp segment (−79 to +38) at the 5′ end of *atp6* is far greater than can be expected by nucleotide substitution alone, given our earlier measurement that the Ogura and normal radish mitochondrial genomes are on average 99.9% identical in nucleotide sequence (13). Indeed, the 848-bp segment immediately following this divergent 118-bp stretch shows 99.8% sequence identity between the two radish genomes (Fig. 2). We postulate that the large number of nucleotide differences in this 118-bp region are the result of ancient duplication events, followed first by a lengthy period of nucleotide divergence and then by recent rearrangement events that led to the replacement of the normal radish −79 to +38 region with the highly divergent region present in Ogura radish. Analysis of the two *atp6* sequences indicates that the 3′ rearrangement occurred 104-bp 3′ to the coding region, just within the *coxI*-

FIG. 7. **Alignment of nucleotide sequences around the 5′ *atp6* rearrangement breakpoint.** The nucleotide sequences from the 5′ *atp6* flanking region of Ogura radish (*O*) and the Ogura *XbaI/ClaI* 0.49-kb fragment (*X*) are shown aligned with the normal 5′ *atp6* radish sequence (*N*). Where the sequences are identical, only that from normal radish is shown. Nucleotides are numbered as in Fig. 2; identical bases are shown as −, missing bases as ·. To facilitate alignment and sequence comparison, 450 bp of the Ogura sequence (nucleotides −552 to −102) are not shown. The deduced sequence for the first 29 amino acids of normal radish is shown below the normal sequence. Where the sequence is different for Ogura, the amino acid is shown; identical amino acids are represented by an *asterisk*. The *trnfM* gene is *boxed*.

homologous sequence present in normal radish *atp6*. This rearrangement event may have occurred by homologous recombination across the *coxI* repeated sequence. No common sequence or structure was identified at both the 5′ and 3′ breakpoints, rather the rearrangements appear to have occurred via homologous recombination across the different repeated sequences found near each rearrangement breakpoint.

DISCUSSION

Rearrangements Associated with atp6—Detailed analyses of *atp6* from both normal and Ogura radish have revealed that the two loci are highly rearranged relative to one another. The sequences that comprise the normal *atp6* locus, including a *trnfM* gene that is co-transcribed with *atp6*, are present in three widely separated regions of the Ogura mtDNA. Similarly, the 3′ flank of Ogura *atp6* is far removed from the *atp6* gene in normal radish, whereas the 5′ flank of Ogura *atp6* is not present in normal radish mtDNA. Within this 5′-flanking region is a 105-amino acid ORF that is co-transcribed with *atp6*.

Although the Ogura *atp6* 5′ flank is not present as an intact unit in the normal radish mitochondrial genome, a 161-bp sequence (Fig. 2, *region B*) from this region has been found in two locations in the normal genome and once in Ogura by DNA sequencing.[3] Furthermore, hybridization data indicate that this sequence may be present in as many as four copies in each of the two genomes. Within the ORF-105 sequence are regions of 44 and 35 bp that are directly repeated a total of two and three times, respectively. Thus, much of Ogura-specific *atp6* DNA consists of short repetitive sequences.

Analysis of the 5′ and 3′ recombination breakpoints failed to identify any specific sequence or structure common to them. However, that repeated sequences, albeit different sets of them, are present at rearrangement sites suggests that they may be involved in homologous recombination events. It is believed that plant mitochondrial genomes exist as mixtures of different sized circular molecules that interconvert via frequent homologous recombination across sets of direct repeats (17, 45). Thus, the rapid structural evolution that plant mitochondrial genomes undergo may also be the result of this recombination, albeit at a much lower frequency.

Despite the highly rearranged nature of the two *atp6* loci, sequences common between them are highly conserved at the DNA sequence level (99.8% identity). This agrees with our earlier findings, based entirely on restriction site mapping, that the two genomes have undergone extensive rearrangement relative to one another, but that unrearranged regions are highly conserved (99.9% identical) at the DNA sequence level (13).

CMS and atp6 Alterations—Our studies suggest two possible ways in which *atp6* could be involved in Ogura CMS: 1) an altered, semifunctional *atp6* polypeptide is synthesized in Ogura mitochondria; 2) synthesis of ORF-105 has a detrimental effect on mitochondrial function. In Ogura radish, the region predicted to encode the 5′ end of *atp6* contains many nucleotide substitutions relative to normal radish. These changes result in the elimination of the proposed ribosome binding site and seven amino acid substitutions, the most critical involving a change from the putative initiator methionine to a leucine codon (Figs. 2 and 3). If our assignment of Met-1 as the start of the normal *atp6* polypeptide is correct, we would predict that if Ogura *atp6* is translated, translation should begin at a different start codon and produce a polypeptide with an altered NH2 terminus.

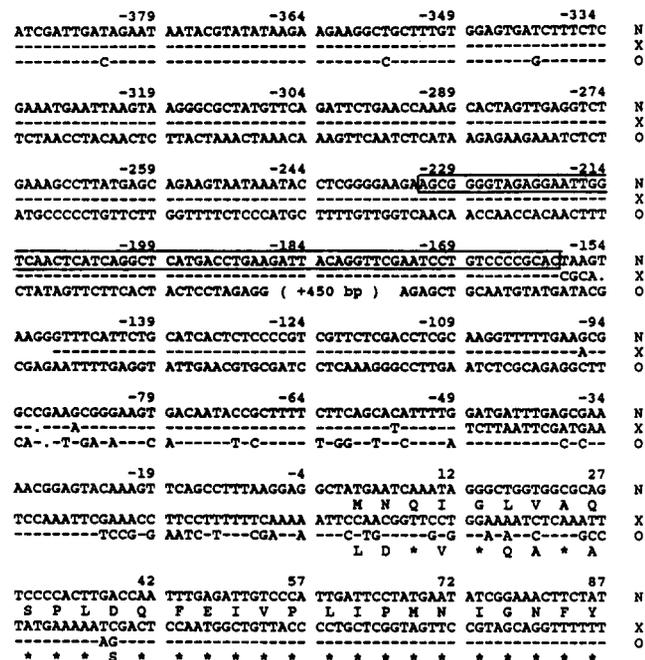Plant *atp6* polypeptides appear to be similar to those of

fungi in that they are synthesized with presequences. NH_2-terminal sequencing of the *S. cerevisiae* and wheat[4] mitochondrial *atp6* polypeptides has identified the sequence S P L E D as the mature NH_2 terminus. We predict that the normal radish polypeptide should be similar to those of *S. cerevisiae* (39) and wheat (38) and should also contain a serine at the NH_2 terminus of the mature polypeptide. There are two candidates for alternate start codons in Ogura *atp6*. The first is an internal methionine (Met-23) and the second is an Ogura-specific GTG codon (nucleotides 10–12). Translation from Met-23 would result in an Ogura protein lacking, at minimum, the first 13 amino acids of the mature polypeptide. Although unusual start codons have yet to be documented in plant mitochondria, they are known to function in animal mitochondria (46) and in *E. coli* (47). Utilization of GTG as the start codon would produce an Ogura *atp6* polypeptide with a shorter presequence and four amino acid differences. If processed correctly, the mature protein would contain only one amino acid substitution: Asp 13 → Ser. Although it has been suggested that the *atp6* presequences may be involved in membrane localization (32), the role of these sequences as well as the identity of residues required for correct processing are unknown. Therefore, if Ogura *atp6* is translated from the GTG codon, it is not clear whether the mutant protein would be localized and processed correctly. Frameshift and nonsense mutations in the *S. cerevisiae oli2* (*atp6*) gene result in respiratory deficient, mit– mutants (48), which can be predicted to be lethal mutations in plants. It is also possible that Ogura *atp6* is not translated, but this seems unlikely.

It should also be noted that although there are several examples of chimeric genes and pseudogenes in plant mitochondria (6, 10, 33, 40, 49), most often there is also a normal copy of the gene elsewhere in the genome. The Ogura radish genome contains a single copy of *atp6*. The only other example of an altered single copy mitochondrial gene is *coxI* in the 9E cytoplasm of sorghum (10). It has been postulated that this alteration of *coxI* may be involved in sorghum CMS (10).

Although subunit 6 of the ATPase is universally encoded by mitochondria, the polypeptide is highly variable in both length and primary sequence. Moreover, analysis of yeast *oli2*– mutants suggests that the protein can tolerate amino acid substitutions at many locations without a gross loss of function (48). Thus, an altered Ogura *atp6* polypeptide may retain sufficient activity for ATP synthesis during most stages of growth and development but fail during microsporogenesis. This idea is supported by cytological and chemical evidence indicating that aborted pollen grains in male-sterile cytoplasms appear, in general, to have starved to death (15, 50).

A second possible role of the Ogura *atp6* locus in CMS could involve ORF-105. In the male-sterile T-cytoplasm of maize, the gene T-*urf13*, generated through a series of rearrangements, has been shown to encode a 13-kDa polypeptide found to be associated with T-toxin sensitivity and male-sterility (6–8). This polypeptide is found associated with the membrane fraction of male-sterile mitochondria and, by analogy to its action in *E. coli* cells, is believed to disrupt mitochondrial respiration through dissipation of the proton gradient (7). The C-terminal half of ORF-105 is hydrophobic and characteristic of membrane-spanning domains. It is possible that ORF-105 is analogous to the 13-kDa polypeptide of maize

CMS-T and also has a detrimental effect on mitochondrial function in Ogura radish.

Nuclear restorer genes have no effect on Ogura *atp6* transcript patterns (13). Therefore, if either the *atp6* polypeptide or ORF-105 are causally related to Ogura CMS, we would expect the restorer genes to act by either 1) supplying a functional ATPase subunit 6 polypeptide, 2) supplying an altered form of a different ATPase subunit to compensate for the *atp6* defect, or 3) blocking the expression or function of ORF-105. Although both *atp6* and ORF-105 are good candidates for being involved with Ogura CMS, further studies, including experiments designed to investigate the ATPase subunit 6 and ORF-105 polypeptides, are necessary to determine what role, if any, they have in this cytoplasmically inherited trait.

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[4] A discrepancy exists between the reported NH_2-terminal amino acid sequence of the mature wheat polypeptide (38) and the amino acid sequence deduced from the *atp6* nucleotide sequence (34). The DNA sequence predicts a threonine residue at the N terminus identified as serine by protein sequencing.

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