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Mitochondrial DNA Rearrangements and Transcriptional Alterations in the Male-Sterile Cytoplasm of Ogura Radish

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Maternally inherited mutations, such as cytoplasmic male sterility, provide useful systems in which to study the function of plant mitochondrial genomes and also their interaction with nuclear genes. We have studied the organization and expression of the organelle genomes of the male-sterile cytoplasm of Ogura radish and compared them with those of normal radish to identify alterations that might be involved in cytoplasmic male sterility. The chloroplast DNAs of Ogura and normal radish are virtually indistinguishable, whereas their mitochondrial DNAs are highly rearranged. Alignment of a restriction map constructed for the 257-kilobase Ogura mitochondrial genome with that published for the 242-kilobase genome of normal radish reveals that the two mitochondrial DNAs differ in arrangement by at least 10 inversions. The transcriptional patterns of several known mitochondrial genes and of rearranged mitochondrial sequences were examined in three nuclear backgrounds. Altered transcripts were observed for three mitochondrial genes, *atpA*, *atp6*, and *coxI*. Rearrangements map near each of these genes and therefore may be responsible for their transcriptional alterations. Radish nuclear genes that restore fertility to the Ogura cytoplasm have no effect on the *atp6* and *coxI* transcripts, but do influence the *atpA* transcriptional pattern.

Cytoplasmic male sterility (CMS) is a maternally inherited trait in which plants fail to produce functional pollen but maintain female fertility (16, 17). The CMS phenotype is often observed when a nucleus from one species is combined with a foreign cytoplasm by backcrossing. Nuclear genes that restore fertility to CMS plants have been identified, indicating that the CMS phenotype results from nuclear-cytoplasmic incompatibility (17, 21). Analysis of the mitochondrial and chloroplast genomes in relation to plant phenotype has demonstrated that CMS is associated with mitochondrial dysfunction (for a review, see reference 24). The mitochondrial genomes of CMS plants exhibit altered organizational properties when compared with those of the related fertile plants (24). In addition, numerous alterations in mitochondrial transcriptional and translational patterns have been associated with CMS in maize (1, 6, 12, 14, 19, 44), sorghum (15), and petunia (46).

The original observation of CMS in crucifers (family *Cruciferae*) was by Ogura (27), who found male-sterile plants of radish (*Raphanus sativa*) in a population of an escaped radish cultivar in Japan. Since then, a number of crucifer CMS cytoplasms have been identified and distinguished from one another by classical genetic tests with restorer alleles (40). The crucifers provide an amenable system to study CMS for several reasons. (i) A large number of crucifer species are sexually compatible in both interspecific and intergeneric crosses, which allows one to examine interactions between nuclear background and cytoplasmic type (3). (ii) Somatic hybrids can be made between *Brassica* species by protoplast fusion and plant regeneration (36). Somatic hybrids between plants with the CMS Ogura cytoplasm and *Brassica campestris* have been shown to contain recombinant mitochondrial DNAs (mtDNAs) (9, 37). (iii) The mtDNAs of *Brassica* species are the smallest and among the best-characterized flowering plant mitochondrial genomes. Complete restriction maps and clone banks exist for several *Brassica* mtDNAs, which range in size from 208 to 242 kilobases (kb) (30, 31, 34). In addition, a detailed transcriptional map that identifies highly expressed regions of the mitochondrial genome has been determined for *B. campestris* (25).

We report here studies of the cytoplasmic genomes of Ogura radish and comparisons with those of normal radish. We show that the two radish chloroplast DNAs (cpDNAs) are identical in organization, whereas the two mtDNAs are highly rearranged. We also compare Ogura mitochondrial transcriptional patterns with those of normal radish. Altered transcripts are identified for three genes. One of these, *atpA*, is affected by nuclear restorer genes.

MATERIALS AND METHODS

Plant material. *Raphanus sativa* cv. Scarlet Knight and the rapid-cycling radish line CrGC7 (Rrr) were used as sources of the normal radish cytoplasm. The rapid-cycling radish line CrGC15 (R1rr) was the source of the Ogura cytoplasm. Sterile and fertile (nucleus-restored) plants containing the Ogura cytoplasm were distinguished by their ability to produce pollen. All rapid-cycling lines (gift of P. Williams and the Crucifer Genetics Cooperative) had been backcrossed to CrGC7 at least six times, implying nuclear homogeneity of at least 99%.

Isolation of nucleic acids. mtDNA was isolated from leaves of 6-week-old plants by the DNase I procedure (23). cpDNA was prepared from these lines by the sucrose gradient procedure (29). mtRNA was isolated in the presence of aurintricarboxylic acid from mitochondria purified by differential centrifugation (42).

Construction of the Ogura restriction map. A clone bank containing Ogura mtDNA *SalI* fragments was constructed in pTZ18, using standard cloning procedures (26). Plasmid DNA was purified by the alkaline lysis miniprep procedure (4). The Ogura restriction map was generated by using the approach of Palmer and Shields (34), hybridizing Ogura and normal radish mtDNA clones to Southern blots containing a series of single and double restriction profiles of the two DNAs. Methods used for restriction endonuclease digestion,

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TABLE 1. Mitochondrial genes mapped in this study

Gene	Protein	Reference
atpA	Alpha subunit of F_1 ATPase complex	7
atp6	Subunit 6 of F_0 ATPase complex	11
atp9	Subunit 9 of F_0 ATPase complex	13
coxI	Cytochrome oxidase subunit I	22
coxII	Cytochrome oxidase subunit II	20
coxIII	Cytochrome oxidase subunit III	22
cob	Apocytochrome *b*	39
18S–5S rRNA		8
26S rRNA		10
ndhI	Subunit 1 of NADH dehydrogenase	43
rps13	Ribosomal protein S13	5

agarose gel electrophoresis of DNA fragments, bidirectional transfer from agarose gels to Zetabind (AMF Cuno) hybridization membranes, labeling of recombinant DNA fragments by nick translation, and filter hybridization were as described previously (29, 30). Gene mapping hybridizations were conducted with cloned fragments (described in reference 25) containing the genes listed in Table 1.

Northern (RNA) hybridizations. RNA (7.5 μg) was electrophoresed in a 1% agarose gel containing 37% formaldehyde–20 mM MOPS (morpholinepropanesulfonic acid), pH 7.0–5 mM sodium acetate–1 mM EDTA and transferred to Zetabind filters in 20× SSC (3.0 M NaCl plus 0.3 M trisodium citrate). Hybridizations were conducted at 60°C for 13 h, using 7.5×10^4 cpm of nick-translated probe per ml of hybridization solution (1 M NaCl, 1% sodium dodecyl sulfate, 5% dextran sulfate). Filters were washed in 2× SSC–0.5% sodium dodecyl sulfate at 60°C prior to fluorography. Chloroplast and cytosolic rRNAs and *Hae*III fragments of phage φX174 were used as size standards. Filters were stripped prior to rehybridization with five changes of a boiling solution of 0.01% sodium dodecyl sulfate in 0.01× SSC.

RESULTS

cpDNA comparisons. Purified cpDNAs from Ogura and normal radish were characterized by analysis of their restriction profiles generated with 30 restriction enzymes that have six base recognition sequences. Of the 30 restriction patterns, 28 were identical, indicating that there has been no major rearrangement of the chloroplast genome in Ogura radish versus normal radish (data not shown). A total of 395 restriction sites representing 2,370 base pairs of normal radish cpDNA were examined. Of these 395 restriction sites, 393 were also present in the Ogura cpDNA. Assuming that each restriction site change is the result of a single-base-pair change, only two nucleotide differences exist between the two genomes in the 2,370 base pairs surveyed. At this level of analysis, these two cpDNAs are practically (99.9%) identical, suggesting that Ogura cytoplasm is derived from within *R. sativa*.

Structure and organization of the Ogura mitochondrial genome. mtDNAs from normal radish and Ogura radish were compared by single and double restriction digests, using the enzymes *Sal*I, *Pst*I, *Bgl*I, and *Nru*I (Fig. 1). Numerous differences in the restriction profiles of the two DNAs are apparent. However, there are no detectable differences in the restriction patterns of Ogura mtDNA isolated from sterile and nucleus-restored backgrounds (data not shown).

To understand the nature of the mtDNA alterations distinguishing the two radish cytoplasms, we constructed a restriction map of Ogura mtDNA and compared it with that published (30) for normal radish (Fig. 2). Clones covering 85% of the Ogura mtDNA, as well as several cloned fragments of normal radish mtDNA, were hybridized to Zetabind filters containing the digests of the two mtDNAs shown in Fig. 1. A map of Ogura mtDNA was constructed by homologous hybridizations and then aligned with the map of normal radish mtDNA on the basis of the heterologous hybridization results (Fig. 2). This alignment shows that the two genomes differ radically in their sequence order. No fewer than 12 linkage groups (where sequences within a group have the same arrangement in the two genomes, but where the relative order and arrangement of the linkage groups differ) are required to align the two genomes. Using the same logic applied in earlier studies for the analysis of cpDNA rearrangements (see Fig. 6 in reference 33 and Fig. 5 in reference 32), we find that a minimum of 10 inversions must be postulated to interconvert the two radish mitochondrial genomes.

An example of the hybridization pattern of a rearranged fragment is shown in Fig. 3A. The 19.4-kb *Sal*I fragment of Ogura mtDNA hybridizes to *Sal*I fragments of 9.8, 9.6, and 3.0 kb in the normal radish genome; these are located in three widely separated regions of this genome (linkage groups 3, 5, and 9; Fig. 2). The *Sal*I-*Pst*I double digestion pattern illustrates that, while many differences are observed for fragments that span boundaries between linkage groups (11.4-kb fragment versus those of 6.5, 5.2, and 3.0 kb), fragments within a linkage group (2.3, 2.0, and 1.0 kb) are conserved in size.

In addition to the rearrangement of homologous sequences, the Ogura mtDNA (257 kb) contains approximately 15 kb of DNA not present in the normal radish mtDNA (242

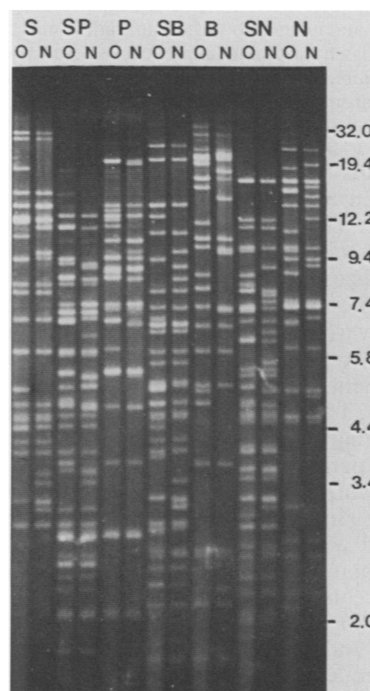

FIG. 1. Restriction analysis of mtDNAs from Ogura (O) and normal (N) radish. DNAs were digested with *Sal*I (S), *Sal*I-*Pst*I (SP), *Pst*I (P), *Sal*I-*Bgl*I (SB), *Bgl*I (B), *Sal*I-*Nru*I (SN), and *Nru*I (N) and electrophoresed on a 0.7% agarose gel. Size scale is in kilobases.

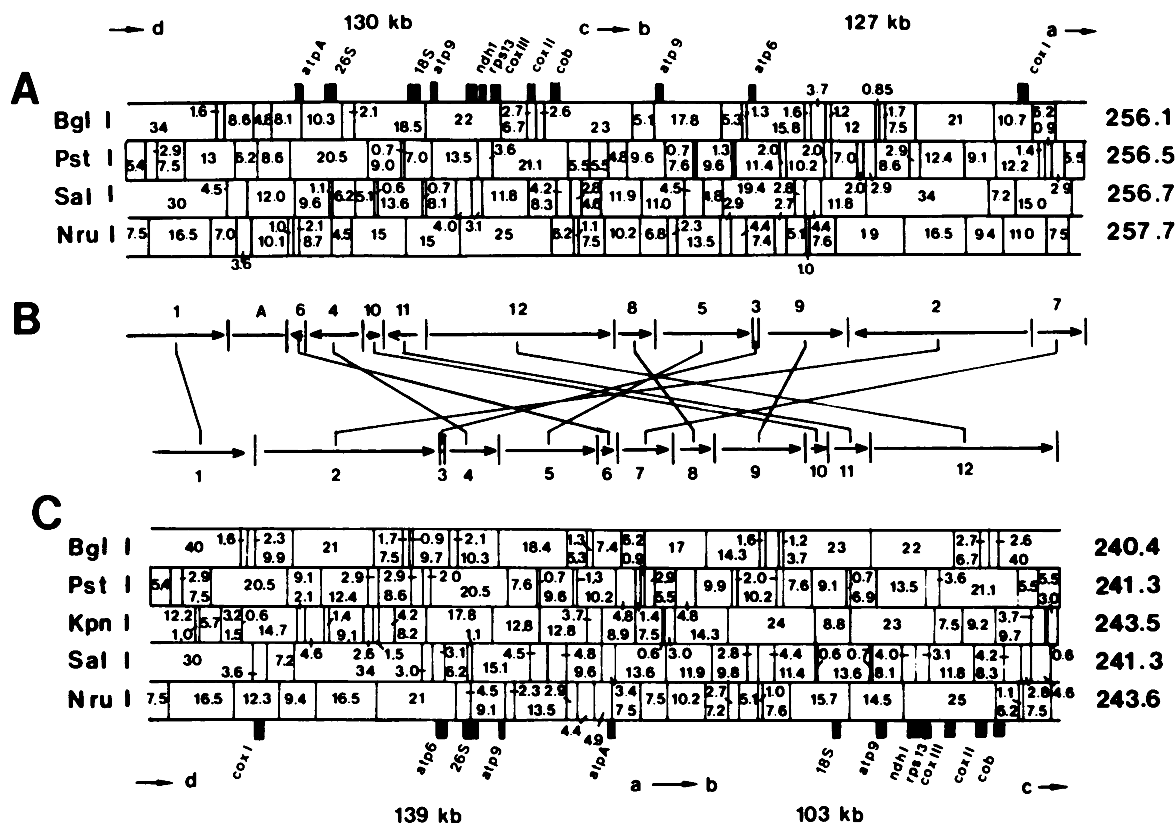

FIG. 2. Comparison of sequence arrangement in the Ogura and normal radish mitochondrial genomes. (A) Restriction site and gene map of the master chromosome of Ogura mtDNA. The circular map is shown linearized at a *Pst*I site internal to the 10-kb repeat. The numbers at the right indicate the summation of restriction fragments for each of the enzymes marked at the left. Arrows indicate the position and relative orientation of the two copies of the 10-kb repeat present on the molecule. Letters denote the unique sequences flanking the repeats, and numbers between them indicate the size of subgenomic circles (cf. Fig. 4). (B) Relative arrangement of homologous sequences in the Ogura (top line) and normal (bottom line) radish mitochondrial genomes. Numbers and arrows indicate the positions and relative orientations of blocks of cross-hybridizing sequences whose arrangement has been conserved between the two genomes. The crossing lines connect homologous sequence blocks. One major block is unique to the Ogura genome and is indicated by "A". (C) Restriction site and gene map of the master chromosome of normal radish mtDNA (30). Labeling conventions used are the same as for panel A.

kb). Most of this "novel" DNA is found in one region of the genome (designated as A), suggesting one major insertion/deletion event. The absence of this region in normal radish mtDNA is illustrated in Fig. 3B. An Ogura *Sal*I fragment of 12.0 kb hybridizes strongly only to Ogura mtDNA (*Sal*I and *Sal*I-*Pst*I fragments of 12.0 kb and 8.6 and 3.2 kb, respectively). However, many weak hybridization signals are seen in both mtDNAs, indicating that the clone contains small repeated elements present in several different locations in both genomes. The 1.7-kb *Bgl*I fragment is an example of a repeat-containing fragment common to both genomes.

Mapping experiments indicate that Ogura mtDNA has a tricircular organization (Fig. 4) similar to that of the normal radish genome (30). The Ogura master circle (257 kb) can be interconverted with subgenomic circles of 127 and 130 kb through recombination within a pair of 10-kb direct repeats. The same repeats are found in normal radish mtDNA, in which a 242-kb master circle coexists with subgenomic circles of 139 and 103 kb (Fig. 4) (30). The arrangement of single-copy sequences that flank the recombination repeats differs between the normal and Ogura radish genomes (Fig. 4). The repeat-flanking sequences a-b and c-d present on the normal radish master circle are located on the subgenomic circles of Ogura mtDNA and vice versa. This arrangement of repeat-flanking sequences in Ogura mtDNA is the same as that seen in *Brassica nigra* mtDNA and probably reflects an analogous series of inversions relative to normal radish as those postulated for *B. nigra* (30).

The positions of known mitochondrial genes (Table 1) were mapped on the normal and Ogura radish mtDNAs by Southern hybridization to ascertain whether any of the rearrangements resulted in gene disruption. Because of the large number of rearrangements, the order of genes in Ogura mtDNA is different from that in normal radish mtDNA (Fig. 2). While several genes, *atp*A, 18S rRNA, *atp9*, and *atp6*, map near inversion breakpoints, a single hybridizing region was identified with each of the heterologous probes with the exception of *atp9*. This gene is present in two copies in both genomes. These results suggest that none of the genes have been duplicated in one genome compared with the other nor has rearrangement resulted in the translocation of a large piece of any gene to a new location. However, translocation of small genic pieces might have gone undetected. It should be noted that, unlike *B. campestris*, which contains two copies of *coxII* and one copy of *atp9* (25, 43), normal and Ogura radish contain two copies of *atp9* and only one copy of *coxII*.

Base sequence divergence in mtDNA. Measurement of evolutionary rates in organelle genomes by restriction mapping indicates that plant mtDNA sequences change very

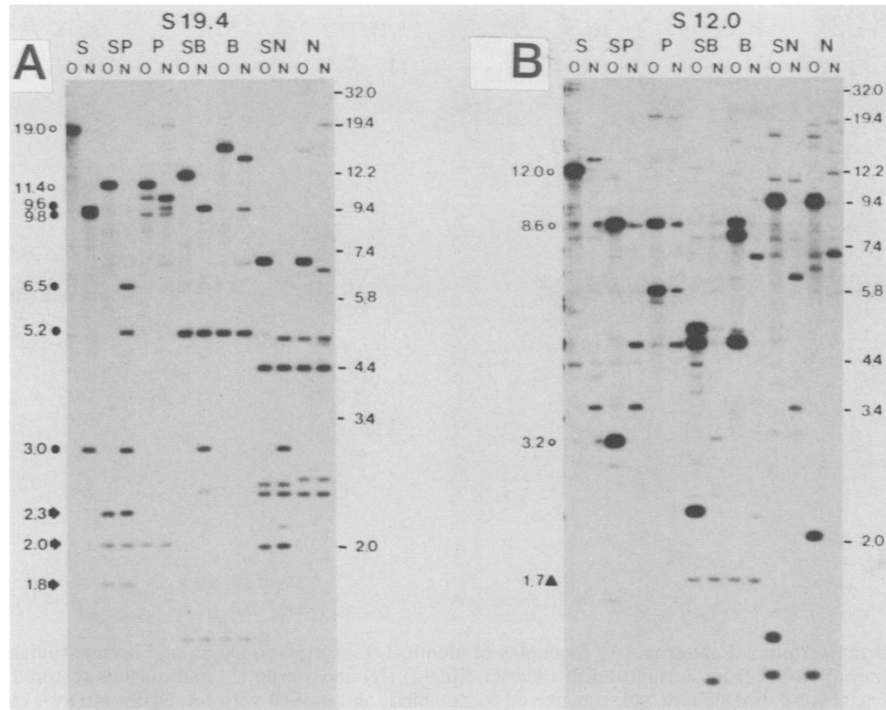

FIG. 3. Detection of mtDNA rearrangements and novel DNA via hybridization between Ogura (O) and normal (N) radish mtDNAs. Two cloned Ogura mtDNA restriction fragments, S19.4 (A) and S12.0 (B), were hybridzed to Zetabind filters containing *Sal*I (S), *Sal*I-*Pst*I (SP), *Pst*I (P), *Sal*I-*Bgl*I (SB), *Bgl*I (B), *Sal*I-*Nru*I (SN), and *Nru*I (N) mtDNA fragments. *Sal*I and *Sal*I-*Pst*I fragments and their sizes are denoted as follows: Ogura specific (open circles); normal radish specific (closed circles); Ogura- and normal radish-common fragments (arrows). A 1.7-kb repeat-containing *Bgl*I fragment is marked with a triangle. Size scales at right are in kilobases.

slowly (31; J. Palmer and L. Herbon, unpublished results). We wished to determine whether the extensive rearrangement of Ogura and normal mtDNAs was accompanied by a corresponding amount of base sequence change. Normal and Ogura mtDNA clones covering 59 kb of unrearranged DNA sequences were digested with 24 restriction enzymes that have six base recognition sequences and then electrophoresed in adjacent gel lanes. Of the 334 restriction sites examined (representing 2,004 base pairs of normal radish mtDNA), 332 were conserved between the two genomes (data not shown). Assuming again that a single-base-pair change is responsible for each restriction site difference, this implies conservation of 2,002 of the 2,004 base pairs of mtDNA sequence compared, or a sequence identity of 99.9%.

Alterations in mitochondrial transcription patterns. Mitochondrial transcript patterns were compared between normal and Ogura radish. Northern (RNA) blots containing mtRNA isolated from sterile or fertile (nucleus-restored) Ogura and from normal radish were probed with Ogura mtDNA clones containing known rearrangements (Fig. 2) and also with mitochondrial gene-containing subclones from *B. campestris*. In most instances, identical transcriptional patterns were observed for all three mtRNAs. Five examples of identical transcripts are shown in Fig. 5A. These include two unidentified transcripts (U), 18S rRNA, and transcripts for *atp9* (identified with S11.0) and *coxII*. (Quantitative variation in levels of identical-size transcripts from *coxII* and *atpA* is the result of differences in the amount of RNA loaded in adjacent lanes.)

Altered transcriptional patterns were observed for three genes, *atpA*, *coxI*, and *atp6* (Fig. 5B). One highly abundant *coxI* transcript (1,900 nucleotides [nt]) is seen in Ogura

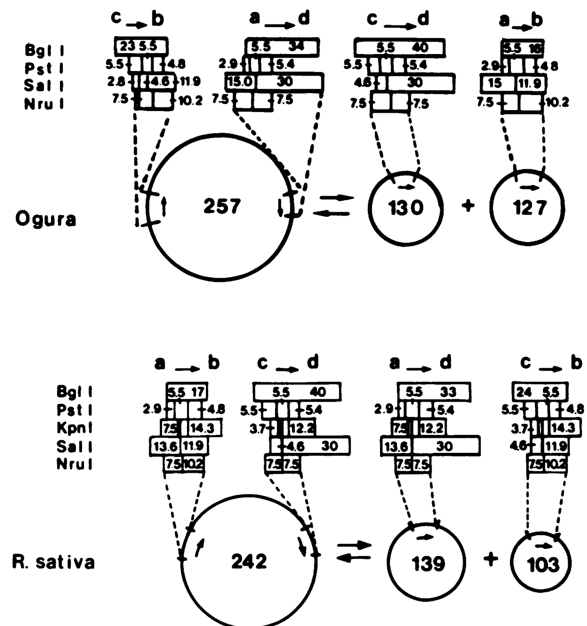

FIG. 4. Tricircular organization of the Ogura and normal radish mitochondrial genomes. Arrows indicate the position and relative orientation of the 10-kb recombination repeats. Letters denote the unique sequences flanking the repeats.

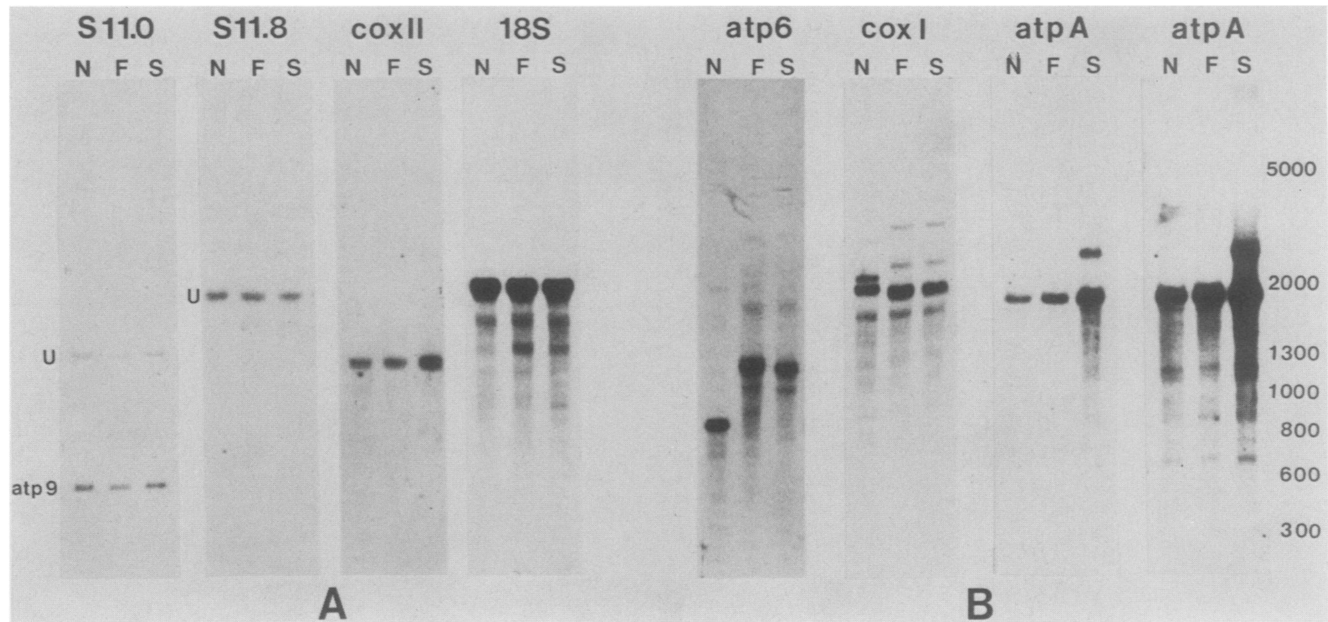

FIG. 5. Mitochondrial transcriptional patterns. (A) Examples of identical transcripts in Ogura and normal radish. (B) Altered transcriptional patterns. Mitochondrial RNAs from normal radish (Scarlet Knight) (N) and sterile (S) and nucleus-restored (F) Ogura radish were electrophoresed on a 1% agarose–formaldehyde gel, transferred to Zetabind, and probed with the Ogura mtDNA clones *Sal*I 11.0 and *Sal*I 11.8 and *B. campestris* clones containing *coxII*, 18S rRNA, *coxI*, *atp6*, and *atpA*. Equal amounts of mtRNA (7.5 μg) were loaded on each lane with the exception of the sterile Ogura radish lanes probed with the *coxII* and *atpA* genes (15.0 μg). Size scale at right is in nucleotides.

radish (both male sterile and male fertile), while two abundant *coxI* transcripts (1,950 and 2,050 nt) are observed in normal radish. Differences are also apparent in the pattern of larger less abundant *coxI* transcripts. The *atp6* transcript is 800 nt in normal radish but 1,200 nt in both Ogura lines. That *atp6* and *coxI* transcripts are identical in sterile and fertile (nucleus restored) Ogura plants (Fig. 5B) indicates the absence of any major effects of nuclear restorer genes on transcription and processing for these two genes. Differences in *atp6* and *coxI* transcript patterns between normal (Scarlet Knight) and Ogura radish could result from either mtDNA alterations or differences in nuclear backgrounds. The *atp6* and *coxI* transcript patterns are identical in mtRNA from the normal cytoplasm of both Scarlet Knight (Fig. 5B) and a rapid-cycling radish line (data not shown) that is isonuclear to the Ogura radish lines analyzed in Fig. 5. Therefore, the *atp6* and *coxI* transcriptional differences are most likely due to mtDNA alterations.

The most abundant *atpA* transcript is 1,800 nt in size in all three radish cytoplasms. However, an additional transcript of 2,700 nt is present in sterile Ogura radish but is absent from both fertile cytoplasms even upon prolonged autoradiographic exposure (Fig. 5B). Thus, unlike the *atp6* and *coxI* genes, whose transcripts vary with cytoplasm but not with nucleus, the *atpA* gene exhibits a nucleus-dependent, cytoplasm-independent pattern of expression.

DISCUSSION

Structural and sequence comparisons of the normal and Ogura cytoplasmic genomes. The cytoplasmic genomes of the male-sterile Ogura radish have been characterized and compared with those of normal radish. The cpDNAs and mtDNAs from the two sources are very similar (99.9% identical) at the sequence level. While the cpDNAs are also identical in structure, the Ogura mtDNA is highly rearranged (differing by at least 10 inversions) relative to normal radish. That no rearrangements and minimal sequence divergence are detected between the two cpDNAs suggests that cpDNA is not responsible for CMS in Ogura radish. This is in agreement with the results of Pelletier et al. (36), who identified the mitochondrion as the source of CMS in the Ogura cytoplasm on the basis of independent segregation of cpDNA and CMS in somatic hybrids.

Several lines of evidence suggest that the Ogura cytoplasm is derived from normal radish and has not arisen through introgression of the radish nuclear genome into the cytoplasm of some other species of *Raphanus* or *Brassica*. (i) Numerous restriction site differences have been found that distinguish the cpDNAs of *R. sativa* and various *Brassica* species (18, 35). The cpDNA divergence observed between Ogura and normal radish (0.10%) is in accord with observed intraspecific values (0 to 0.20%) and not with interspecific values (0.30 to 2.6%). (ii) Recombination repeats appear to be generated and lost quite readily in plant mtDNA (30, 31), yet Ogura and normal radish mtDNAs share the same 10-kb repeat element. (iii) Very little sequence divergence is observed in unrearranged regions of the two mitochondrial genomes.

Alignment of the restriction map reported here for Ogura mtDNA with that published (30) for normal radish reveals that sequences common to the two genomes are extensively rearranged. A minimum of 10 inversion events must be postulated to account for the cross-hybridization results. The extent of mtDNA rearrangement between the two radish cytoplasms exceeds that found between many pairs of *Brassica* species and is at the extreme for the genus (J. Palmer and L. Herbon, unpublished results).

In addition to the large number of inversions and rearrangements that distinguish the two radish mtDNAs, the configurations of the two direct repeats located on the master chromosome of the Ogura mtDNA are present on the subgenomes of normal radish and vice versa. This is similar

to the situation in *B. nigra* relative to normal radish (30) and implies two inversion events that are independent of any repeat-mediated recombination.

The high degree of rearrangement present in Ogura mtDNA relative to normal radish mtDNA appears to be a common feature of CMS plants. To our knowledge, every CMS mtDNA examined to date exhibits altered restriction patterns when compared with its normal counterpart, and, when examined further, these alterations have been shown to result from rearrangements (2, 12, 18, 21, 24, 46).

Organization and expression of Ogura mitochondrial genes. The arrangement of mitochondrial genes in Ogura and normal radish is quite different as a result of the extensive rearrangement documented in Fig. 2. Rearrangement endpoints map in close proximity to the genes *atpA*, 18S rRNA, *atp9*, *atp6*, and *coxI* (Fig. 2). Multiple rearrangements have had the effect of moving two small gene-containing blocks (*atpA* and *atp6*) to another region of the genome. The precise positions of rearrangements relative to genes have not been determined. Southern analysis indicates that there has been no major disruption of these genes. However, point mutations, small deletions, and rearrangements at the end of a gene would not have been detected, and therefore gene alterations cannot be ruled out.

Mitochondrial gene alterations are associated with CMS in maize, sorghum, and petunia. Rearrangements in the CMS-T cytoplasm of maize result in a novel gene containing portions of the flanking or coding regions of the 26S rRNA, *atp6*, and chloroplast $tRNA^{Arg}$ genes (12). This gene encodes a 13-kilodalton protein whose presence and abundance correlate with both CMS and toxin sensitivity (14, 38, 44, 45). In sorghum, a mitochondrial genome rearrangement possibly associated with CMS in the 9E cytoplasm results in the altered transcription and translation of the *coxI* gene (2). An open reading frame resulting from the fusion of parts of *atp9*, *coxII*, and an unidentified reading frame is associated with CMS in petunia (46).

We find that three radish genes that map near mtDNA rearrangements exhibit altered transcript patterns in Ogura radish compared with normal radish. The altered transcript patterns can be divided into two classes. Alterations of *atp6* and *coxI* transcripts are the result of mtDNA differences and are independent of nuclear background. Transcripts from *atpA*, on the other hand, are affected by nuclear background but not by mtDNA type. Although nuclear restorer genes have no effect on the *atp6* or *coxI* transcript pattern, these mitochondrial genes could still be involved in CMS if the restorer genes act at stages other than transcription or RNA processing.

In contrast to the situation for *atp6* and *coxI*, radish nuclear restorer genes are associated with differences in the pattern of *atpA* transcripts. In addition to an 1,800-nt transcript present in all three lines examined in Fig. 5, sterile Ogura radish, which lacks nuclear restorer genes, contains an additional transcript (2,700 nt) in fertile lines containing both the Ogura and normal radish cytoplasms. This raises the possibility that the 2,700-nt transcript may be associated with male sterility. While the nature of the radish nuclear restorer genes is unknown, our data suggest that at least one may be involved in RNA processing. It has been suggested that one or both of the maize nuclear restorer genes RF1 and RF2 are involved in RNA processing (12).

We have not determined whether functional *atpA*, *atp6*, and *coxI* proteins are synthesized in the mitochondrion in male-sterile Ogura radish. While further studies are required to determine whether any or all of these genes are causally related to CMS in Ogura radish, our work in conjunction with previous studies (2, 12, 14, 38, 44–46) illustrates the broad range of mitochondrial alterations found in CMS plants and suggests that each system will have a unique set of alterations.

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