Tricircular mitochondrial genomes of *Brassica* and *Raphanus*: reversal of repeat configurations by inversion

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ABSTRACT

We constructed complete physical maps of the tripartite mitochondrial genomes of two Crucifers, *Brassica nigra* (black mustard) and *Raphanus sativa* (radish). Both genomes contain two copies of a direct repeat engaged in intragenomic recombination. The outcome of this recombination in black mustard is to interconvert a 231 kb master chromosome with two subgenomic circles of 135 kb and 96 kb. In radish, a 242 kb master chromosome interconverts with subgenomic circles of 139 kb and 103 kb. The recombination repeats are 7 kb in size in black mustard and 10 kb in radish, and are nearly identical except for two insertions in the radish repeat relative to the black mustard one. The two repeat configurations present on the master chromosome of black mustard are located on the subgenomes of radish and vice-versa. To explain this, we postulate the existence of an evolutionarily intermediate mitochondrial genome in which the recombination repeats were (are) present in an inverted orientation. The recombination repeats described for these two species are completely different from those previously found in the closely related species *B. campestris* (1), implying that such repeats are created and lost frequently in plant mitochondrial DNAs and making it less than likely that recombination occurs in a site-specific manner.

INTRODUCTION

Three hallmarks of plant mitochondrial DNAs (mtDNAs) are their inexplicably large and variable sizes (2), their possession of copious quantities of chloroplast DNA sequences (3-5), and their existence as a collection of different-sized circles that interconvert via recombination across pairs of direct repeats (1,6-10). In certain species, such as turnip (1) and spinach (7,8), only a single pair of recombination repeats is present per genome equivalent. These mitochondrial genomes exist in a tricircular form in which a master chromosome containing the entire sequence complexity of the genome interconverts with two smaller subgenomic circles, each bearing one copy of the repeat. A larger number of recombination repeats is found in mtDNAs of maize (6) and wheat (7,9,10); consequently these genomes have much more complex multicircular arrangements.

In order to further our understanding of the range of complex forms

exhibited by plant mtDNAs and of the recombination repeats that generate these forms, we have determined the physical organization of mtDNA from two members of the crucifer family, Brassica nigra (black mustard) and Raphanus sativa (radish). Previous studies (2,7) had shown that mtDNAs of distantly related taxa are too divergent in size and form for meaningful comparison. Therefore, in order to produce a proper time frame for studying the evolution of genome form, we chose to study these two closely related (11) crucifers, and to compare them to the previously characterized B. campestris (1; turnip). Furthermore, we were interested specifically in knowing the structure of mtDNA in radish in order to allow future study of mtDNA alterations in the male sterile cytoplasm of this species (12), and as a basis for understanding the novel mitochondrial genomes that arise out of protoplast fusions involving this cytoplasm (13).

We show here that the mitochondrial genomes of radish and black mustard have similar tripartite structures. However, although the recombination repeats in these two species are very similar to each other and almost certainly originated by the same event, they are completely different from the sequence that is repeated and recombining in turnip (1). Moreover, the radish and black mustard repeats differ with respect to the configuration of unique sequences that flank them on the master chromosome and subgenomic circles.

MATERIALS AND METHODS

MtDNA was isolated from six week old green leaves of black mustard (Brassica nigra 84/row 1307; gift of D. Cohen), radish (Raphanus sativa cv. Scarlet Knight), and turnip (B. campestris cv. Purple Top White Globe) by the DNAase I procedure (14). Clone banks of mtDNA PstI fragments from black mustard and radish were constructed by ligating PstI-digested mtDNA with PstI-digested plasmid pUC8 (15), transforming into E. coli strain JM 83 (16), and selecting recombinant (white) colonies on X-gal indicator plates. Plasmid DNAs were purified by the alkaline miniprep procedure (17). Uncloned mtDNA PstI fragments used as hybridization probes were isolated from preparative agarose gels by electroelution, followed by phenol extraction, ether extraction, and ethanol precipitation. Methods used for restriction endonuclease digestion, agarose gel electrophoresis, bidirectional transfer of DNA fragments from agarose gels to Zetabind (AMF Cuono) hybridization membranes, labeling of recombinant plasmids and isolated fragments by nick-translation, and filter hybridization were as described (18,19). Filters were stripped of hybridized probe prior to rehybridization by incubation in 0.5 M NaOH at 50° for one hour.

RESULTS**Mitochondrial DNA Recombination Repeats**

We constructed restriction maps of the mitochondrial genomes of black mustard and radish using the approach described in our mapping study on the mtDNA of turnip, *Brassica campestris* (1). In brief, clone banks containing about 70% of each mtDNA were constructed by inserting mtDNA PstI fragments into the plasmid pUC8. Each clone was then used in a mapping experiment by labelling it with ^{32}P and hybridizing to a Zetabind filter containing digests of the appropriate mtDNA with PstI, SalI, BglI, KpnI, NruI, PstI-SalI, PstI-BglI, PstI-KpnI and PstI-NruI. Also, each uncloned PstI fragment was isolated from a preparative gel, nick-translated, and hybridized to a similar filter.

This set of mapping hybridizations revealed a single major repeat element in each genome. A 3.3 kb PstI fragment from black mustard, which lacks sites for both BstEII and BglI, hybridizes to four fragments in BstEII-BglI digests of total mtDNA from both black mustard and radish (Fig. 1). Restriction maps of these four fragments reveal a central common region, which is approximately 7 kb in length in black mustard (Fig. 2A) and 10 kb in radish (Fig. 2B). Each

Figure 1. Recombination repeats in *Brassica* and *Raphanus* mtDNAs. Purified mtDNAs from *B. nigra* (N), *R. sativa* (R) and *B. campestris* (C) were digested with BstEII-BglI and PstI, electrophoresed in a 0.7% agarose gel, transferred to a Zetabind filter, and hybridized with nick-translated BN3.3, a plasmid containing the 3.3 kb PstI fragment internal to the 7 kb recombination repeat of *B. nigra*, and BC4.8, a plasmid containing a 4.8 kb EcoRI fragment that contains the entire 2 kb recombination repeat from *B. campestris*. Sizes of hybridizing fragments are indicated in kb.

Figure 2. Restriction maps of the four repeat-containing, substoichiometric restriction fragments from black mustard (A) and radish (B) mtDNAs. Numbers at left of maps indicate the size of the repeat-containing fragments resulting from BstEII-BglI (BG) digestion (cf. Fig. 1), while numbers and letters at right indicate the sizes and flanking sequence configurations of the BglI (G) fragments from which the double digest fragments are derived (cf. Figs. 3 and 4). Cleavage sites shown: BstEII (B), PstI (P), NruI (N), SalI (S), KpnI (K), BglI (G), SacI (C), HindIII (H) and SmaI (A). All sites are shown for the first six of these enzymes, but only selected sites for the last three. The solid bars indicate the minimum extents of the repeats and open extensions of the bars their maximum extents. (C) Comparison of recombination repeats and flanking sequences c and d. Asterisks denote restriction sites unique to one of the two fragments compared. Filled triangles indicate insertions in the radish repeat relative to the black mustard repeat.

repeat-containing fragment is identical on one side of the repeat to one of the other three fragments, and on the other side of the repeat to a second one of the other three (Fig. 2). In other words, the repeat in each genome is flanked by four paired combinations of four unique sequences. The largest and smallest fragments (e.g. of 22 kb and 13 kb in the case of black mustard) in each set contain the same sequences as the intermediate-sized pair (of 19 kb

and 16 kb) and the two pairs of fragments are interconvertible by a single reciprocal cross-over within the central repeat element (Fig. 2).

These observations parallel those reported for mtDNAs from several other species, which also contain repeated elements active in recombination (1,6-10). We hereby term the 7 kb black mustard and 10 kb radish repeats "recombination repeats" in accord with the definition previously used (7) to denote a sequence that is present in two copies relative to the rest of the genome, but which exists in four different genomic environments as the result of recombination between repeats. Note that, as predicted by this definition, each repeat-containing fragment is present in substoichiometric amounts (roughly half-molar) relative to all other, unique fragments (Fig. 1; this is true for all but the 16 kb fragment of black mustard, which comigrates with a unimolar unique fragment). This reflects the fact that each repeat-containing fragment is present on only one of two genomic isomers, whereas unique fragments are present on both forms. Taking into account degradation effects, fragment stoichiometries suggest that the four repeat-containing fragments in each genome are present in equal amounts relative to one another and at half the level of other fragments (Fig. 1). This indicates that the two genomic isomers are present in equal amounts, and suggests that recombination between the two forms occurs at a very high frequency.

The recombination repeats in black mustard and radish are largely identical (Figs. 1 and 2). Alignment of the repeats based on cross-hybridizations and restriction site homologies reveals common borders (as defined by PstI sites at the left end and SacI sites at the right end; Fig. 2C). The 3 kb size difference between the two repeats reflects primarily two internal insertions/deletions. Factoring out these two length mutations, we find that eight of nine restriction sites internal to the repeats are conserved between the two species (Fig. 2C). Furthermore, colinearity and restriction site homology continue into both unique flanking sequences in the particular repeat configuration compared in Fig. 2C.

The nearly identical sequences that are repeated and recombining in the mtDNAs of Brassica nigra and Raphanus sativa are present in only one copy in B. campestris mtDNA (Fig. 1). Conversely, a 2 kb sequence element that is duplicated and recombining in B. campestris (1) is present only once in the other two species (Fig. 1). Thus, completely different (at the level of filter hybridizations) sequences are duplicated and engaged in intragenomic recombination in the mtDNAs of these closely related species. Furthermore, the 2 kb B. campestris recombination repeat contains the gene for cytochrome

	→ d 96 kb														c → b 135 kb														a →
Bgl I																													230.5
Pst I	2.9	1.6			2.4	1.1	10.8	2.8	8.7	2.6	4.2	3.9	16.0	5.3	1.6	1.2	1.5	1.7	21	3.2	10.2	6.2	3.2	229.7					
Kpn I	4.6	7.7	2.3	5.3	3.6	32	13.9	8.9	9.8	5.2	6.9	8.7	7.0	15.0	1.3	3.7	2.0	0.9	2.9	12.4	5.7	12.2	2.3	230.9					
Sal I	12	1.5	11.3	6.5			16.0	5.5			6.1	21	15.0	9.5	10.7	1.0	2.0	8.5	12.4	5.7	12.2	2.3	231.9						
Nru I	11.5	7.6	7.1	7.4	15.8	1.6	4.5	2.8	2.3	14.8	8.0	26	6.1	2.0	23	8.2	9.2	14.8	8.6	2.9	0.5	12.5	5.3	231.1					

[illegible]

oxidase subunit II (7), so that changes in gene copy number accompany the creation and loss of these repeats.

The mapping experiments described in the preceding section allowed the construction of circular restriction maps which contain the entire sequence complexity of the black mustard and radish mitochondrial genomes (Fig. 3; for convenience, the circular maps are shown in a linear form). The black mustard genome is 231 kb in size, only slightly smaller than the 242 kb radish genome. The distances between recombination repeats within each genome are also very similar in the two species (Fig. 3). Most critically, in both genomes the two repeats have a direct orientation relative to one another. Consequently, recombination between repeats causes each master circle to resolve into two smaller, subgenomic circles, each bearing a single repeat element (Fig. 4). Based on the equimolar stoichiometry of the four repeat-containing fragments (Fig. 1; see above), the three circles are judged to be present at the same

Figure 4. Tricircular organization of the *Brassica nigra* and *Raphanus sativa* mitochondrial genomes. Arrows indicate the position and relative orientation of recombination repeats. Letters denote the sequences flanking the repeats.

Figure 5. Evolutionary model for the interconversion of the *Raphanus sativa* and *Brassica nigra* mitochondrial genomes. Arrows indicate the position and relative orientation of recombination repeats. Dashed lines connect endpoints of hypothetical inversions. Letters denote homologous sequences in the two genomes that flank the repeats (cf. Figs. 3 and 4).

copy number. The tricircular form of these two mitochondrial genomes closely resembles those previously described for turnip (1) and spinach (8).

The four unique sequences flanking the recombination repeats are homologous in the black mustard and radish genomes. This is diagrammed in Fig. 2C for the two flanks designated "c" and "d", and was demonstrated by heterologous filter hybridizations in the case of the "a" and "b" flanks (data not shown). Interestingly, the two flanking sequence combinations (a-d and c-b) on the master chromosome of black mustard are present on the subgenomic circles of radish, and vice-versa (Fig. 4). The simplest way to interconvert two such genome arrangements requires a minimum of three inversions (Fig. 5). In temporal order, the first is an inversion occurring during evolution that flips one repeat relative to the other, thereby generating an inverted orientation of the two repeats. The second is a repeat-mediated inversion, analogous to the "flip-flop" inversions of yeast 2-micron DNA (20) and chloroplast DNA (21). The third is another evolutionary inversion, which occurs in the "flipped" orientation of the chromosome and which inverts a repeat, thereby regenerating a direct orientation of the repeats (Fig 5). In other words, in a tripartite genome with direct repeats, the repeat configurations of the master chromosome and subgenomic circles can be exchanged only by going through an intermediate genome with inverted repeats, in which "flip-flop" recombination can interconvert two master chromosomes, one bearing one pair of repeat configurations and the other the second pair (Fig. 5).

Discussion

Our mapping studies reveal that the overall size and form of the mitochondrial genome is quite similar in black mustard and radish. Both mtDNAs contain a single major repeat element, which is present in two directly oriented copies. High frequency recombination between repeats generates a tricircular arrangement of each mtDNA. In black mustard, a 231 kb master chromosome interconverts with subgenomic circles of 135 kb and 96 kb. In radish, the circle sizes are 242 kb, 139 kb and 103 kb, respectively. The three circles are present in equimolar amounts in both genomes.

These gross similarities in genome structure belie, however, a fundamental divergence in linear sequence arrangement. One form of evidence for this rearrangement is the observation that the two repeat configurations present on the master chromosome in one species are located on the subgenomes of the other, and vice-versa. This implies the occurrence in the lineages leading to these two species of two evolutionary inversions that occurred independently

of any repeat-mediated recombination. Further evidence comes from attempts to manually align the black mustard and radish mtDNA maps. These efforts are largely fruitless and imply, in the face of a high degree of primary sequence homology (Fig. 2C; also cf. ref. 11), substantial rearrangement. Finally, in studies to be reported elsewhere, we have directly compared the linear arrangement of sequences in these two and several other closely mtDNAs, and have documented upwards of two dozen evolutionary inversions. These findings extend previous, more indirect reports (22,23) of high rates of rearrangement in plant mitochondrial genomes.

The recombination repeats in black mustard and radish are largely identical in sequence, have the same ends, and are embedded in the same two regions of the mitochondrial genome. Therefore, it is likely that they originated by the same duplication event. In contrast, a completely different sequence is repeated and recombining in the closely related, and also tricircular, turnip mitochondrial genome (1). Furthermore, we have recently identified a third Brassica genome type with respect to recombination. This is the mtDNA of B. hirta (white mustard), which lacks any recombination repeats and therefore exists as a single circle (24). These findings extend an earlier study (7) and suggest that recombination repeats can be created and lost relatively rapidly in plant mtDNAs. Large-scale changes in genome form mediated by repeat-sequence interactions have also been seen in the S male sterile cytoplasm of maize. Here, recombination between the ends of two S-specific linear plasmids and homologous sequences in chromosomal mtDNA converts the normally circular chromosomes into linear ones (25).

The occurrence of different recombination repeats among closely related species makes a site-specific model for recombination somewhat less than likely. Such a mechanism could still operate, but would require either different recombination sites in each repeat (and presumably different enzymes to operate at these sites) or else multiple copies of the same site spread throughout the genome. Furthermore, such sites would have to be small enough as to go undetected in our hybridization studies. Therefore, we presently favor a generalized mechanism (26) of plant mtDNA recombination over a site-specific model suggested earlier (1,7,9).

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