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Mitochondrial genome structure of rice suspension culture from cytoplasmic male-sterile line (A-58CMS): reappraisal of the master circle

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Summary. The mitochondrial DNA (mtDNA) from the cultured cells of a cytoplasmic male-sterile line (A-58CMS) of rice (*Oryza sativa*) was cloned and its physical map was constructed. There was structural alteration on the mitochondrial genome during the cell culture. Detailed restriction analysis of cosmid clones having mtDNA fragments suggested either that the master genome has a 100-kb duplication (the genome size becomes 450 kb) or that a master circle is not present in the genome (the net structural complexity becomes 350 kb). The physical map of plant mitochondrial genomes thus far reported is illustrated in a single circle, namely a master circle. However, no circular DNA molecule corresponding to a master circle has yet been proved. In the present report, representation of plant mitochondrial genomes and a possibility for mitochondrial genome without a master circle are discussed.

Key words: Cytoplasmic male sterility – Cultured cell – Rice mtDNA – Physical map – Master circle

Introduction

Plant mitochondrial genomes have been intensively investigated on a molecular basis to clarify the mechanism of cytoplasmic male sterility (cms), because several observations indicate that a major cytoplasmic factor of the cms trait resides in mitochondria rather than in chloroplasts. Plant mitochondrial genomes are larger than the mitochondrial genomes of any other organisms that have been studied to date. In addition, there are generally heterogeneous molecular species in plant mitochondria. The heterogeneity of plant mitochondrial genomes arises from their multipartite structure and the presence of several kinds of plasmids. These characteristics make it difficult to determine the genome size and the entire gene organization.

Although the mitochondrial genome has a heterogeneous structure, its physical map has been constructed for a number of plant species (Palmer and Shields 1984; Lonsdale et al. 1984; Chétrit et al. 1984; Stern and Palmer 1986; Palmer and Herbon 1986, 1987; Siculella and Palmer 1988; Fauron et al. 1989; Folkerts and Hanson 1989). It has been reported that the mtDNA of *Brassica campestris,* for which one of the first physical maps of plant mtDNA was determined, has a single pair of direct repeats, and that recombination between these repeats results in the division of the original circular molecule (218 kb) into two smaller molecules (135 and 83 kb). Consequently, the mitochondrial genome of *B. campestris* is believed to consist of three molecular species, and this structure is termed a tripartite structure (Palmer and Shields 1984). This genome model seems to work sufficiently well in much more complex genomes with a large number of repeated sequences active in homologous recombination. Subgenomic molecules generated by homologous recombination contain a certain region of the master circle (or master chromosome) (Lonsdale et al. 1984). The master circle therefore represents the entire complex of the DNA molecules that are not able to generate a further subgenomic molecule. However, it is still unknown whether or not the master circle in multipartite genomes really exists as a single DNA molecule, because no large circular DNA molecule corresponding to the master circle has been directly detected in mitochondria of higher plants by electron microscopic observations or

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other methods (Bendich 1985; Bendich and Smith 1990). Rapid rearrangement of mitochondrial genome induced by prolonged cell culture or somatic cell fusion suggests that plant mitochondrial genome is in a dynamic equilibrium of homologous recombination (Lonsdale et al. 1988).

The model of a master circle and multipartite structure is apparently general and is an attractive explanation of the heterogeneous mixture of plant mtDNA. Further analyses, however, suggest that there are molecules of very low abundance which cannot be explained by recombination between major repeated sequences. Such substoichiometric molecules, termed sublimons, have been examined most intensively in maize (Small et al. 1987). The function and origin of sublimons are entirely obscure, but it is speculated that they have played an essential role in creating a rearranged genome in the course of evolution (Small et al. 1989). An identical mechanism has also been proposed in the case of regeneration of cms-T maize (Fauron et al. 1990). These authors regard the sublimons as a transition form of the genome structure by intermolecular recombination among subgenomic molecules, resulting in the elimination of a certain region from the original genome.

We have cloned the mtDNA from cultured cells of a cms line (A-58CMS) of rice (*Oryza sativa*) and have constructed its physical map. The data presented in this paper reappraise the conventional model, which holds a single master circle to be the norm of the mitochondrial genome of higher plants.

Materials and methods

Rice lines

The A-58CMS line has an *Indica*-type cytoplasm of the Chinsurah Boro II line on a *Japonica*-type nucleus of A-58 (a line suitable for relatively cold districts), and it therefore exhibits alloplasmic cms. Cultured cells in suspension derived from the A-58CMS and the Chinsurah Boro II lines were used as sources of mtDNA (Shikanai et al. 1987). Total DNA was prepared from leaves and shoots of the intact plants (A-58CMS).

DNA preparation and cloning

The isolation of mtDNA was performed as described by Shikanai et al. (1987) with a slight modification: we treated mitochondrial suspension in lysis buffer with proteinase K at 65 °C for 5 min prior to lysis.

To construct a mitochondrial genomic library, the isolated mtDNA was partially digested with *Mbo*I restriction endonuclease and ligated with the *Bam*HI digests of a cosmid pHC79 for A-58CMS mtDNA, and of a cosmid pWE15 for Chinsurah Boro II mtDNA, after size fractionation of *Mbo*I digests by sucrose gradient ultracentrifugation (Maniatis et al. 1982). The ligates were packaged using Gigapack Gold (an *in vitro* packaging kit of λ-DNA) (Stratagene), then infected to *E. coli* HB101 or DH1 strain for A-58CMS mtDNA, and *E. coli* NM554 strain for Chinsurah Boro II mtDNA, respectively. The cosmid colonies obtained were transferred to microtiter plates for long-term storage and to nylon membrane (Biodyne A, Pall) for colony hybridization.

The cetyltrimethylammoniumbromide (CTAB) method (Lichtenstein and Draper 1985) was used to isolate total DNA from the leaves of intact A-58CMS plants and from the shoots of A-58CMS grown in the dark of 10 days at 25 °C.

Hybridization analysis

DNA preparation was digested with restriction endonuclease and resolved by electrophoresis in an 0.6% agarose gel. The DNA fragments fractionated in size were transferred to a nylon membrane (Biodyne A or Biodyne B, Pall) by the conventional capillary method. Hybridization was done at 42 °C in hybridization buffer (50% formamide, 5 × Denhardt's solution, 6 × SSC, 0.5% SDS, and 0.2 mg/ml sonicated and denatured calf thymus DNA) withg a ^{32}P-labelled probe prepared by random priming reaction (a kit for the reaction was purchased from Boehringer Manheim). When hybridization was completed, the membrane was washed successively in 2 × SSC containing 0.1% SDS at room temperature, in 1 × SSC and 0.1% SDS at room temperature, and in 0.5 × SSC and 0.1% SDS at 65 °C. Autoradiography was usually performed at room temperature with an X-ray film (Konica), but at −70 °C with an intensifying screen when hybridization signals were faint.

Results

The restriction profiles of cosmid clones

Although it has been reported that tissue culture causes structural alteration of mitochondrial genome in plants (McNay et al. 1984; Rode et al. 1987; Chowdhury et al. 1988; Dörfel et al. 1989; Shirzadegan et al. 1989), we used the cell line in suspension culture as a source of mtDNA for two reasons; (1) the seeds of A-58CMS are of limited availability, (2) cultured cells must retain the mitochondrial function necessary to maintain proliferation of cells. The isolated mtDNA from the cultured cells of line A-58CMS was digested with several restriction endonucleases. The mtDNA exhibited highly complicated restriction profiles (Fig. 1). Although multimolar restricted fragments make the size estimation difficult, the mitochondrial genome size was calculated at approx. 300 kb by summing up the sizes of the fragments.

The recombinant molecules containing a branching point

The *Xho*I, *Sma*I, and *Kpn*I restriction profiles of the obtained cosmid clones were compared with one another to establish overlaps between the individual clones. The cosmid library can be assumed to contain the whole mitochondrial genomic DNA, because this library consists of all the major DNA fragments generated by digestion with the *Xho*I, *Sma*I, and *Kpn*I restriction endonucleases. The probes used to locate genes by Southern hybridization are summarized in Table 1.

A number of divergence points (branching points) due to homologous recombination were observed on the restriction map of rice mtDNA during its construction

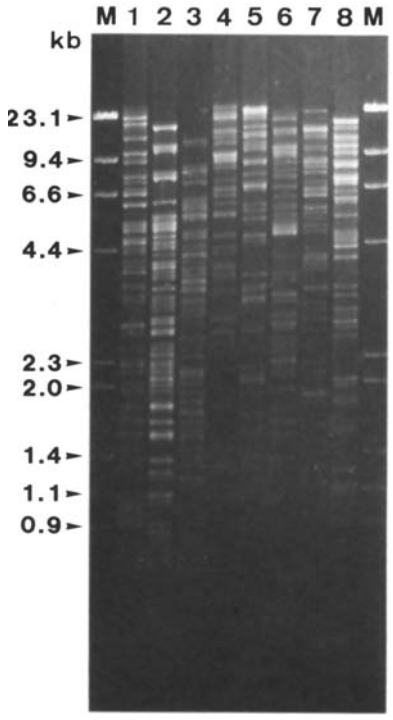

Fig. 1. Restriction profile of mtDNA prepared from the cultured A-58CMS. Mitochondrial DNA was digested with a respective restriction endonuclease: lane *1*, *Bam*HI; lane *2*, *Eco*RI; lane *3*, *Hin*dIII; lane *4*, *Kpn*I; lane *5*, *Pst*I, lane *6*, *Sac*I; lane *7*, *Sma*I; and lane *8*, *Xho*I. Lane *M* shows size markers (kb) of *Hin*dIII-digested λDNA and *Hae*III-digested φX174 DNA

Table 1. A list of known genes used for Southern hybridization

Gene	Source	Reference
atp6	*Oenothera* 1.5-kb *Nhe*I fragment	Schuster und Brennicke (1987)
atpA	Pea 1.5-kb *Eco*RI-*Hin*dIII fragment	Morikami and Nakamura (1987)
cob	Wheat 0.7-kb *Hin*dIII-*Bam*HI fragment	F. Quétier, personal communication
coxI	*Oenothera* 0.6-kb *Bam*HI-*Eco*RI fragment	Hiesel et al. (1987)
coxII	*Oenothera* 0.8-kb *Hpa*I-*Pst*I fragment	Hiesel and Brennicke (1983)
coxIII	*Oenothera* 1.1-kb *Eco*RI-*Pst*I fragment	Hiesel et al. (1987)
nad1	*Oenothera* 1.1-kb *Bam*HI-*Eco*RI fragment	A. Brennicke, personal communication
rps13	*Oenothera* 1.0-kb *Eco*RI fragment	A. Brennicke, personal communication
rrn18	Wheat 1.1-kb *Ava*I fragment	F. Quétier, personal communication
rrn26	Pea 1.8-kb *Eco*RI fragment	K. Nakamura, personal communication

with the cosmid library as described by Coulthart et al. (1990). Inserts of cosmid clones having a branching point can be attributed to either *in vivo* DNA recombination between homologous sequences or to cloning artifacts. However, it was possible to discriminate the latter by Southern hybridization of a DNA fragment of a cosmid clone having a branching point to the total mtDNA digests or to the digested DNA from other cosmid clones (Lonsdale et al. 1986). Cosmid clones with a branching point were isolated. DNA probes having a gene (*rrn18*) for a ribosomal 18S RNA showed multiple hybridized bands to the mtDNA digests of eight different restriction endonucleases (Fig. 2). This indicates that the *rrn18* gene exists as a multicopied gene, which was confirmed by comparing the physical maps of the cosmid clones isolated by colony hybridization with the *rrn18* probe (Fig. 3). There are three species (1A, 2A, and 3A in Fig. 3) of flanking sequences on one end of the common, ca. 2-kb region containing *rrn18* gene, and four species (1B, 2B, 3B, and 4B in Fig. 3) on the other end of the flanking region. In fact, 11 of 12 possible recombinant sequences were obtained as cosmid clones (Table 2); the remaining sequence must then be present in the mitochondria. Further analyses of cosmid clones revealed the presence of another recombination site close to the 2-kb region

Table 2. Cosmid clones containing various configuration of *rrn18* gene

	1B	2B	3B	4B
1A	18S17	HB55	18S24	18S13
2A	18S14	18S29	18S43	18S10
3A	*	18S5	18S25	Sm4.0-1

* A cosmid clone was not obtained (see also Fig. 3)

(*rrn18* gene) resulting in an additional flanking sequence (sequences 4B and 2B in Fig. 3). All of the four possible sequences on this recombination site were obtained, and it was confirmed by hybridization analysis that these recombinant sequences result from homologous recombination (data not shown). Therefore, the 2-kb region (*rrn18* gene) has three kinds of flanking regions on each end, indicating that there are three copies of *rrn18* genes in the rice mitochondrial genome, although it is not clear whether or not all the *rrn18* genes are transcribed. A three-copied *rrn18* gene has been found in the rye mitochondrial genome, where detailed physical mapping showed the presence of an outer and an inner repeat (Coulthart et al. 1990) and also in the wheat mitochondrial genome (F. Quétier, personal communication). The

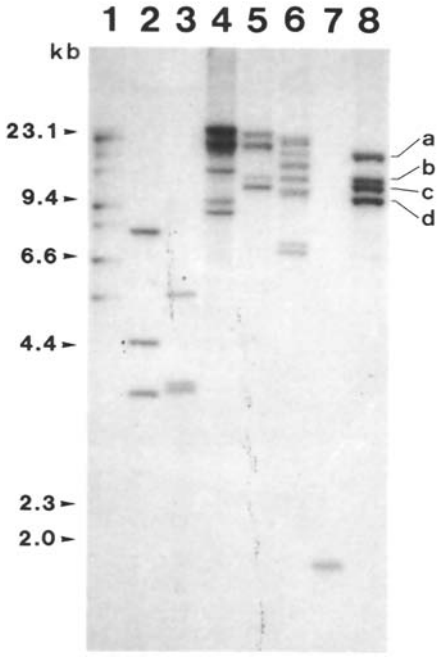

Fig. 2. Southern analysis of *rrn18* gene. DNA fragments shown in Fig. 1 were transferred to a nylon membrane and hybridized with a ^{32}P-labelled probe containing a portion of wheat mitochondrial *rrn18* sequence. The lanes are identical to those of Fig. 1. In lane 7, mtDNA digested with *Sma*I showed a single band because this fragment is completely within a repeated sequence. All hybridization signals detected in *Kpn*I (lane 4), *Sma*I (lane 7), and *Xho*I (lane 8) can be attributed to the physical maps shown in Fig. 3. For example, the *Xho*I fragments (lane 8) with lowercase letters (a, b, c, and d) correspond to the *Xho*I fragments shown in Fig. 3

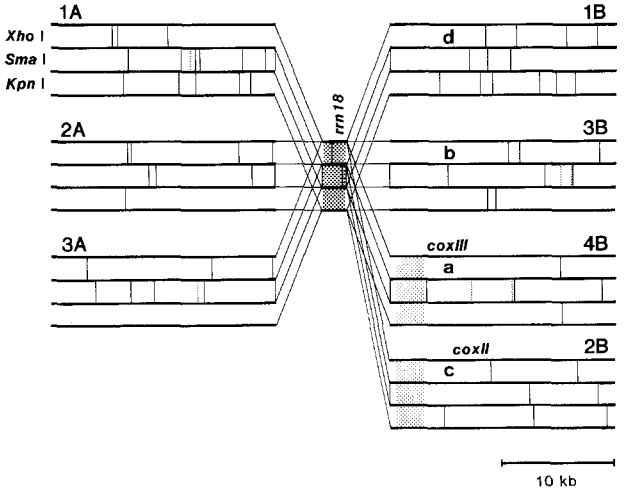

Fig. 3. Physical maps surrounding the *rrn18* gene. The *heavily stippled* region indicates a repeated sequence containing the *rrn18* gene and the *lightly stippled* region indicates another repeated sequence. The orders of fragments separated by *dotted lines* were not determined. The lowercase letters (a, b, c, and d) correspond to the hybridization signals shown in Fig. 2

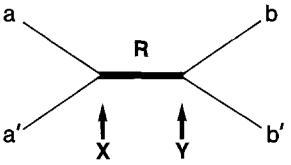

Fig. 4. Two branching points due to a pair of repeats. A diagram showing two branching points (X and Y) on the ends of the repeat (**R**) indicates the existence of four possible recombinant clones (a-R-b, a-R-b', a'-R-b, and a'-R-b')

rice mitochondrial genome is likely to possess such an *rrn18* gene configuration, indicating the close evolutionary relationship within the family Gramineae. A three-copied repeat was also found in Petunia and nine possible recombinant sequences were detected (Folkerts and Hanson 1989).

As described earlier, a set of recombinationally active repeats can have two branching points (X and Y in Fig. 4). For example, branching point X has its partner Y with the repeat (R), indicating the existence of four kinds of recombinant sequences due to homologous recombination. In fact, two clones were obtained and the physical maps around the *atpA* gene are shown in Fig. 5 A. The Southern hybridization with a probe of pea *atpA* gene to total mtDNA also showed two fragments having *atpA* genes corresponding to different clones (see the *Sma*I-digested fragments in Fig. 5 B). The branching point (corresponding to the branching point Y in Fig. 4) shown by a vertical line with a horizontal arrow was expected to be a product of homologous recombination due to repeated sequence near the *atpA* gene. This implies that another branching point (corresponding to the branching point X in Fig. 4) must exist somewhere in the direction shown by an arrow in Fig. 5 A. To detect a partner of the branching point (so-called hidden branching point), the whole DNA of individual cosmid was labeled with ^{32}P-dCTP and hybridized to the total mtDNA digests of *Xho*I, *Sma*I, and *Kpn*I. Then, any hidden branching points could be visualized as extra restriction fragments, which are different from the restriction fragments of the cosmid DNA used as a probe (Fig. 6). However, we could not observe any signals indicating a hidden branching point in the region (approximately 60 kb long, see the dashed line under linear map in Fig. 9) from the branching point near the *atpA* gene to the branching point due to the *rrn18* repeat. However, one hidden branching point was indicated to exist near the *atp6* gene upon performing the same experiments mentioned above (data not shown).

Comparison of mitochondrial gene organization from the cultured cell and the intact plant

As described earlier, tissue culture can induce quantitative and qualitative alterations on plant mtDNA, and a

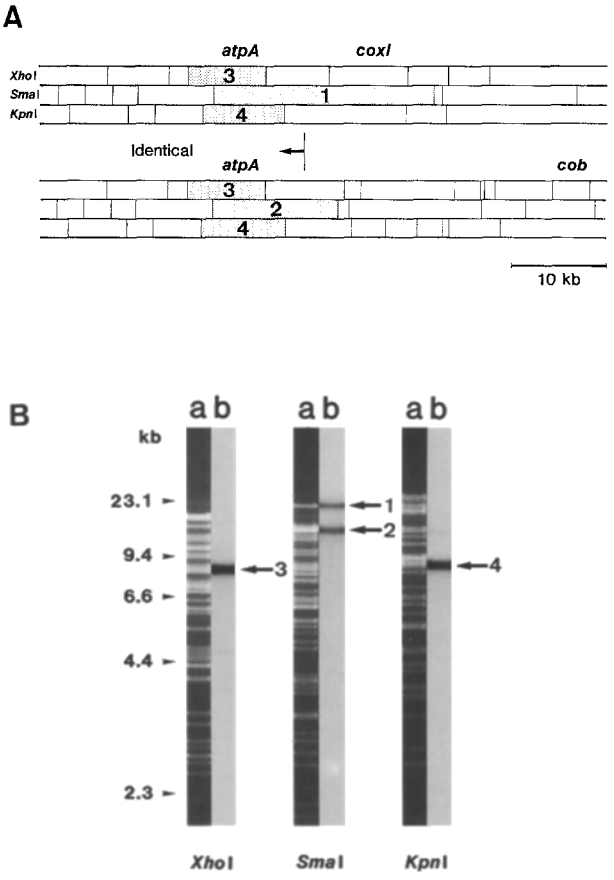

Fig. 5 A–B. A branching point adjacent to the *atpA* gene. **A** Physical maps of two cosmid clones containing the *atpA* gene. The approximate locations of the *atpA* gene on the maps are shown. The *stippled* fragments (**1, 2, 3,** and **4**) correspond to the hybridization signals indicated with *arrows* in **B**. A vertical between the two maps is an approximate position of a branching point, and the regions on the left of the vertical line are indistinguishable in the physical map. **B** Restriction profiles (lane *a*) and hybridization signals (lane *b*) of the *atpA* gene. A probe containing a portion of the pea *atpA* gene (Morikami and Nakamura 1987) was hybridized to the *Xho*I, *Sma*I, and *Kpn*I digests of mtDNA

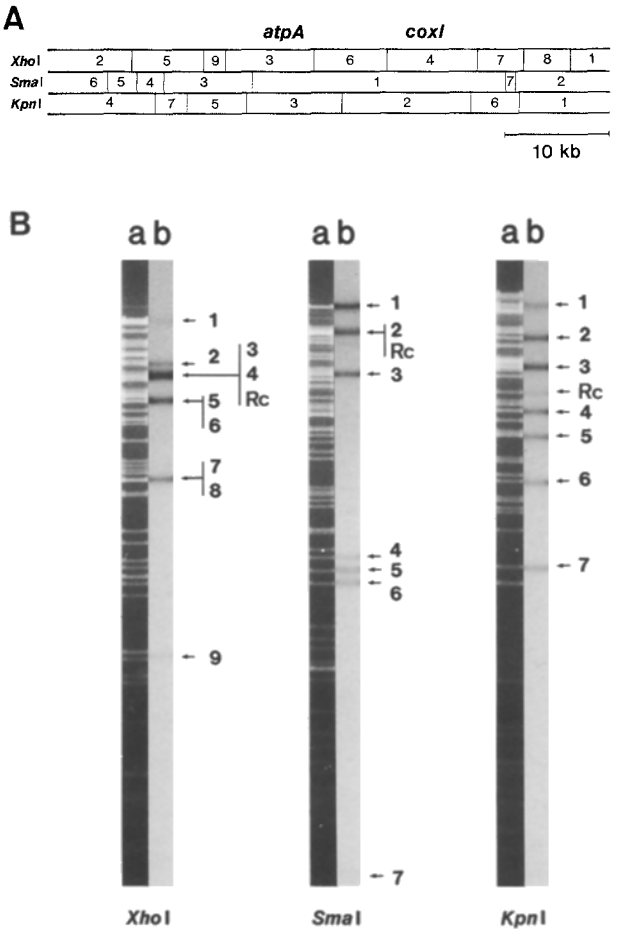

Fig. 6 A and B. Hybridization of a cosmid DNA (HB76, see Fig. 9) to the total mtDNA digests of the cultured A-58CMS. **A** A physical map restricted with *Xho*I, *Sma*I, and *Kpn*I of the mtDNA inserted in the cosmid clone (HB76) used as a probe. **B** Mitochondrial DNA fragments digested with *Xho*I, *Sma*I, and *Kpn*I (lane *a*) and hybridization signals (lane *b*) with a probe (^{32}P-labeled HB76 DNA). Numbers correspond to fragments shown in **A**. ***Rc*** indicates mtDNA fragment having the same branching point due to homologous recombination (see physical maps of clones in Fig. 5A)

nuclear genotype can also affect the structure of mitochondrial genome (Mackenzie et al. 1988; Escote-Carlson et al. 1990). We attempted to determine whether the hidden branching point of the cultured A-58CMS mtDNA is present in the mtDNAs of A-58CMS intact plant or in those of other rice strains. A comparison was made between the mtDNAs prepared from the cultured A-58CMS line, the intact plant (leaves and shoots) of the A-58CMS line, and the cultured Chinsurah Boro II line. The A-58CMS line has a cytoplasm of the Chinsurah Boro II on the nuclear background of the A-58 line. Therefore, all three types of cell lines must have identical cytoplasmic traits. Hybridization analyses showed a difference of *cob* gene configuration between the three sources of mtDNA. The intact plant of A-58CMS and the cultured Chinsurah Boro II had two types of *cob* genes, but the cultured A-58CMS gave a single band of the *cob* gene (Fig. 7B). This indicates that the exctra *cob* gene was lost in the cultured A-58CMS in the course of cell culture, although the cultured Chinsurah Boro II retained it.

To determine the correlation between the hidden branching points and the lost gene, cosmid clones with the *cob* gene were isolated from the library of the cultured Chinsurah Boro II line. Two types of cosmid clones with the *cob* sequence were obtained from the mtDNA of the cultured Chinsurah Boro II, as expected (Fig. 7A). One type is indistinguishable in the physical map of the cultured A-58CMS (Fig. 7A, upper), and the other is not found in the cultured A-58CMS (Fig. 7A, lower). The second *cob* (*cob'*) gene from a different rice line with a cytoplasm of Chinsurah Boro II has been reported to be

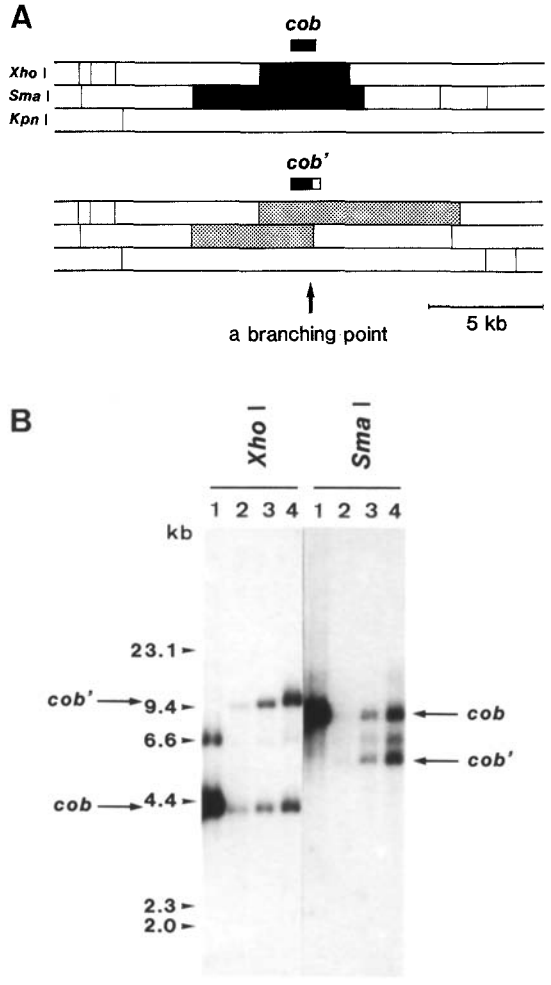

Fig. 7. **A** Physical maps of two mtDNA regions of the cultured Chinsurah Boro II containing a sequence homologous to the *cob* gene. The positions and lengths of the *cob* and *cob'* genes (presented as a *box* above each physical map) were based on the data published by Kadowaki (1989) and Kaleikau et al. (1990). A *closed box* shows the *cob* sequence and an *open box* shows an unidentified reading frame. Hatched restriction fragments correspond to hybridization signals shown in **B. B** Southern hybridization of the wheat *cob* gene to mtDNAs from the cultured A-58CMS (lane *1*), total DNAs from mature leaves (lane *2*) and shoot (lane *3*) of A-58CMS plant, and mtDNA from the cultured Chinsurah Boro II (lane *4*). The amount of DNA applied to lane *1* was much greater than those in lane *2*, and lane *3*, and lane *4* to detect the *cob'* band seen in the other lanes. Signals at 6.6 kb may be nonspecific

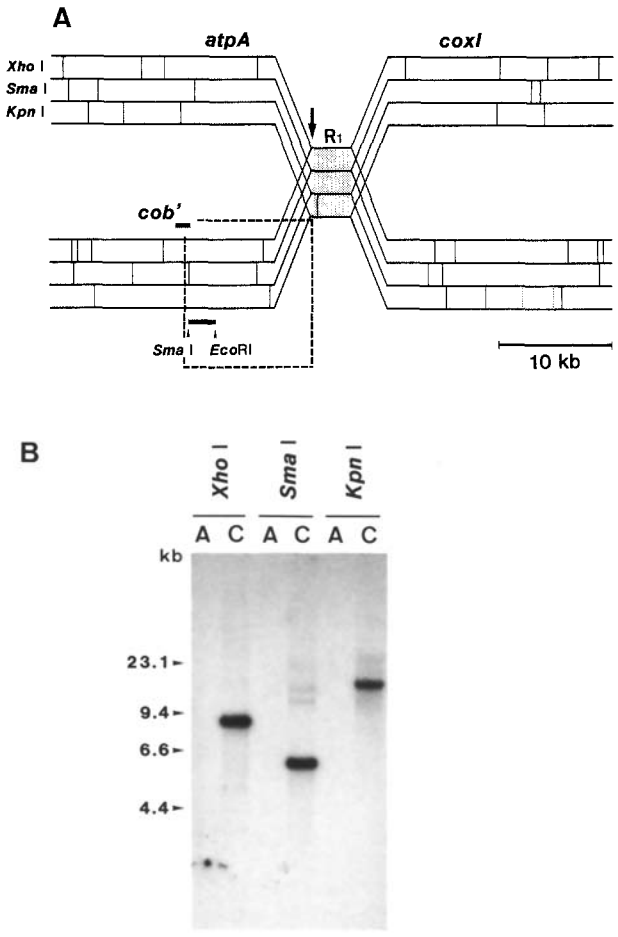

Fig. 8. **A** Physical maps around a repeat (R_1, a *stippled* region; its exact length is not determined) found in the cultured Chinsurah Boro II. The 8-kb region is shown by a *stippled box*. **B** Southern hybridization of a *cob'*-specific fragment to mtDNAs from the cultured A-58CMS (lane *A*) and from the cultured Chinsurah Boro II (lane *C*). An *Sma*I-*Eco*RI 2.3-kb fragment of pOSB376 provided by Kadowaki (1989) was used as a probe (see the *bar* below the map). *Arrows* indicate a hidden branching point in mtDNA of the cultured A-58CMS

a chimeric gene containing almost the whole 5' portion of the *cob* gene flanked by an unidentified reading frame (Kadowaki 1989). A cosmid clone with the *cob'* gene from the cultured Chinsurah Boro II was found to have the *coxI* gene, including a roughly 8-kb region between the *cob'* and the *coxI* gene which was not mapped on the cultured A-58CMS mtDNA (Fig. 8A, the boxed region defined by dashed lines). A portion of the 8-kb region (2.3-kb *Sma*I-*Eco*RI fragment shown in Fig. 8A) was hybridized to the mtDNA digests of the cultured A-58CMS and the cultured Chinsurah Boro II. This result showed that the 8-kb region was missing from the mtDNA of the cultured A-58CMS (Fig. 8B). Therefore, the point from which the 8-kb region is derived corresponded to the hidden branching point in the cultured A-58CMS mtDNA (arrow in Fig. 8A).

Overall physical map

We determined the entire structure of the mitochondrial genome through restriction analysis of cosmid clones. Figure 9 shows a linear physical map of the cultured A-58CMS mtDNA with an approximate length of 350 kb. The ends of the map are not linked to one another because cosmid clones HB76 and HB22 on the ends of the overall physical map extend into another region of the map (cf. the *atpA* and *atp6* regions in Fig. 9, respec-

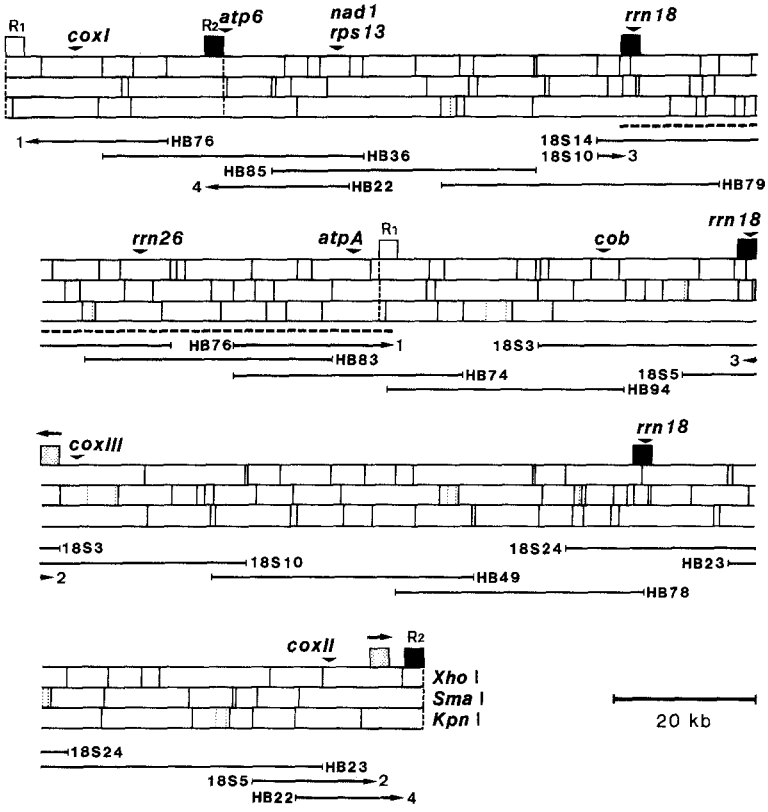

Fig. 9. The overall physical map of mtDNA of the cultured A-58CMS. Approximate locations of genes are shown by *arrowheads* with the genetic symbols. A minimal number of cosmid clones are shown by *bars* with their names below the physical map, each *bar* showing a region covered with a cosmid clone. The orders of fragments separated by *dotted lines* in the map are not determined. Due to homologous recombination, cosmid clones HB22, HB76, 18S5, and 18S10 have two separate regions on the map (*bars* with *arrow* and respective numbers). *Dashed line* below the map indicates the region searched for a hidden branching point for the repeat, R_1 (see text). A variety of boxes above the map indicates the locations of sets of repeated sequences. All sets of repeated sequences except those with *arrows* are oriented in same direction. *Vertical dashed lines* at the repeated sequences of R_1 and R_2 indicate arbitrary locations of a hidden branching point

tively). A cosmid clone, HB22, was found to have another branching point due to homologous recombination at the repeated sequence (R_2). One of the branching points was hidden, therefore we could not obtain any clone separating from one end of the repeat (R_2). Cosmid clones 18S5 and 18S10 also extend into other regions due to homologous recombination at the repeated sequences containing the *rrn18* gene. As described in the previous section, a portion of the DNA fragment (the 8-kb region in Fig. 8A) filling the gap between the ends (R_1 and R_2 in Fig. 9) was found in the mtDNA of the cultured Chinsurah Boro II by restriction analysis and Southern hybridization. Another branching point derived from the 8-kb region in a certain region of the physical map is required for the construction of a circular map (a master circle) in the mitochondrial genome of the cultured Chinsurah Boro II. To detect the branching point in the cultured Chinsurah Boro II mtDNA, the same hybridization experiments as described in Fig. 6 were performed on the Chinsurah Boro II mtDNA by using cosmid DNAs of the cultured Chinsurah Boro II as probes. However, the remaining gap containing the branching point due to a hypothetical repeat (R_2 in Fig. 9) was not found in the cultured Chinsurah Boro II mtDNA (data not shown). This implies the possibility that the single master circle was absent even from the mitochondrial genome of the Chinsurah Boro II line.

Discussion

It is conventionally preferred to construct a plant mitochondrial genome into a single master circle. Most mitochondrial genomes of higher plants investigated to date are reported to be circular, possessing all the genetic contents. However, no direct evidence for the circularity of mtDNA has been presented. Our results showed the lack of a mtDNA fragment of the cultured A-58CMS line derived from the intact plant. A similar phenomenon was reported in common bean at the restoration of fertility (Mackenzie and Chase 1990). Taking into account these results, construction of a single circular map with all

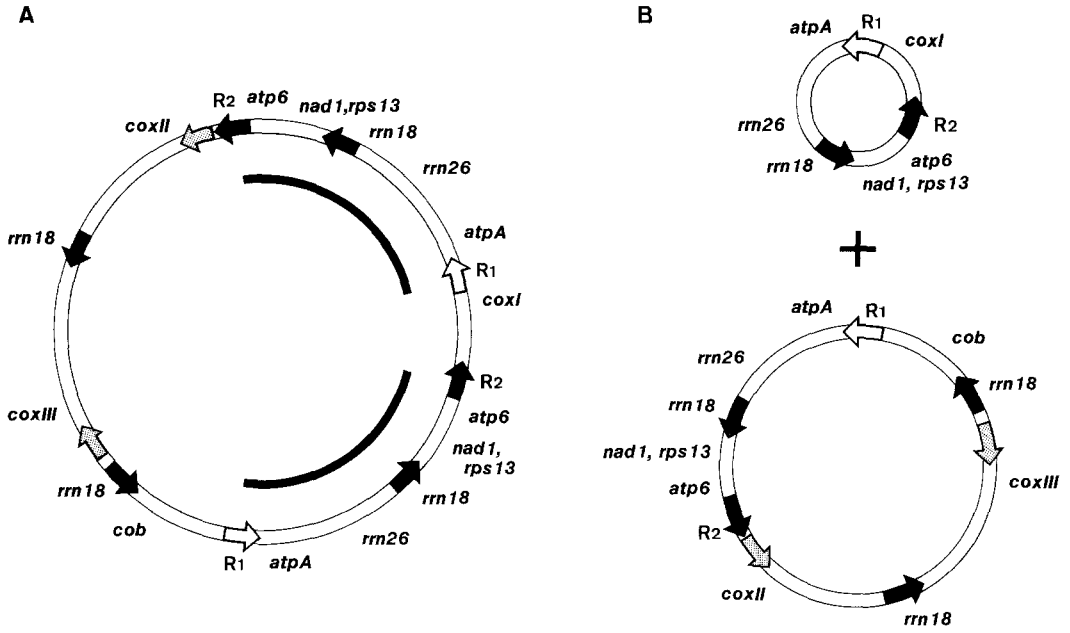

Fig. 10 A and B. The overall structure of mtDNA of the cultured A-58CMS. A variety of boxes with *arrows* indicates the sets of repeated sequences. *Arrows* show relative orientation of the repeated sequences. **A** A circular genetic map with large duplicated regions shown by *thick arcs*. **B** An example of the dicircular model representing the whole mt genome

cosmid clones requires an approximately 100-kb duplication that results in generation of a fourth *rrn18* gene (located in the thick arcs in Fig. 10 A). On the other hand, two circular maps can be introduced to represent the whole genome to avoid the large duplication in the map (Fig. 10 B). An elimination-duplication model for a single circle has been reported for two types of maize mitochondrial genome, the N genome (Small et al. 1989) and the V3 genome, derived from the cultured cms-T cell (Fauron et al. 1990). The mitochondrial genome of the cultured A-58CMS is very similar to the V3 genome because the duplicated regions of the V3 (165 kb) and the cultured A-58CMS (100 kb) are simply introduced into the respective genomes to make a circular map (thick arcs in the circle in Fig. 10 A). It is unclear whether or not such duplications actually exist in the genomes, because the repeated sequences are too long (over 100 kb) to be cloned within a single cosmid. The validity of a physical map with such duplications is not certain. A DNA molecule corresponding to a master circle must be demonstrated by a physico-chemical method in order to construct the physical map of a single master circle. However, the duplications observed in the cultured A-58CMS, the N, and the V3 genomes may be introduced by a similar generation mechanism, regardless of the lengths of duplications (approx. 100 kb in the cultured A-58CMS, 12 kb in the N, and 165 kb in the V3). Therefore, if evidence of the 12-kb repeats in the N genome is demonstrated by the cloning of the DNA fragment containing the entire repeated sequences (12 kb), the duplication model for the other two genomes is possible.

Lonsdale et al. (1984) described the existence of 12-kb direct repeats and several other repeats on the N master circle, and Small et al. (1989) explained the occurrence of duplication of the 12-kb repeat containing the entire *atpA* sequence, with a similar mechanism of duplication applied to the generation of the V3 genome. However, neither the cosmid clone having the full length of the 12-kb repeat and both of its flanking regions in their master circle are obtained, nor are the hybridization data indicating the presence of recombinant molecules presented. It is either possible that cloning of the DNA segments is very difficult for an unknown reason or that the DNA segments are in fact absent from the DNA population of the N mitochondrial genome. Small et al. (1989) demonstrated the generation of the N master circle by combining two subgenomic circles derived from the master circle of an ancestral maize (RU), but the N master circle lacks a specific region for the RU genome.

Bendich and Smith (1990) are not in favor of the circularity of plant mtDNA and the existence of a master circle (a unit genome) as a genetic entity. Lonsdale et al. (1988) have mentioned that the actual status of plant mtDNA is difficult to judge. Insofar as there is no appropriate technique for direct examination of DNA population and structure in higher plant mitochondria, a master circle can be a hypothetical molecule containing no more than a minimum of complete sequence complexity, i.e., net genetic content in plant mitochondria. Therefore, the mitochondrial genomes of the N maize, the regenerated cms-T maize, and the cultured A-58CMS line cannot be demonstrated as a single circle; rather the mitochondrial

genomes must be shown as a linear genome with two ends of distinct repeats that caused elimination of the region between the repeats (Fig. 9). On the other hand, there can be a dicircular model where a genome comprises two subgenomic circles with a large duplication, if closed molecular feature or circularity of physical structure is definitely requested. Both of these representations seem to be insufficient compared with the conventional unicircular structure, but they are much more convincing in describing the structure of plant mitochondrial genome. The data described here cannot exclude the presence of a molecule corresponding to a master circle, but do suggest the dispensability of a master circle as a molecular genetic entity.

How genetic integrity is maintained in apparent molecular fluidity of plant mitochondria is a matter of concern. Mitochondrial DNA in some organisms has been reported to be associated with the inner membrane, especially in the case of a slime mold, *Physarum polymorphalum* (Kuroiwa 1982). A complex is formed site-specifically with a membrane that may play a role in partitioning mtDNA in dividing mitochondria (Kawano and Kuroiwa 1985). Fluorescence microscopic analysis revealed that mtDNA of *Allium cepa* exists as a nucleoid in a mitochondrion and segregates evenly into two daughter mitochondria during mitochondrial kinesis (Nishibayashi and Kuroiwa 1985). A mitochondrial nucleoid of yeast consists of nucleic acids and several proteins, one of which is basic and is thought to bind to mtDNA (Miyakawa et al. 1987). Considering these facts, we can speculate as to how plant mitochondria maintain their genetic integrity. If a master circle is assumed, only its replication and distribution seem to be necessary and sufficient for the maintenance of genetic integrity, although a strict mechanism for protecting the master molecule from homologous recombination is required. If a protein complex to retain all the multipartite DNA molecules is assumed, a master circle molecule is not necessary to the preservation of genetic information. If genes for proteins in the complex are nuclear encoded and under control of the nucleus, structural alteration of mitochondrial genomes observed in cultured cells could be explained by substantial alteration of nuclear gene expression and organization induced by culturing procedure (Scowcroft and Larkin 1988). Furthermore, it is necessary for an extensively divided genome, a multipartite form, without a master molecule to have respective repliction origins for amplification of the whole genome. Otherwise, a multipartite form without DNA replication origin can be extinguished, but no direct evidence of multiple replication origins has been obtained so far. On the other hand, active homologous recombination may be an alternative process for the proper DNA amplification to avoid the elimination of mtDNA segments. As discussed above, it is possible for a mitochondrial genome to exist in a multipartite form without a master circle.

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