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Alteration of mitochondrial genomes containing *atpA* genes in the sexual progeny of cybrids between *Raphanus sativus* cms line and *Brassica napus* cv. Westar

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Summary. We have investigated the fate of the mitochondrial genomes of cybrids derived from "donor-recipient" protoplast fusion between X-irradiated *Raphanus sativus* (cms line) and iodoacetamide-treated *Brassica napus* cv. Westar. Two out of ten fusion products were male-sterile with the diploid chromosome number of *B. napus*. The mitochondrial (mt) genomes of the cybrids and their progeny were further analyzed by DNA-DNA hybridization using the pea mitochondrial ATPase subunit gene (*atpA*) as a probe. One cybrid, 18-3, had a 3.0 kb fragment characteristic of *B. napus* and a 2.0 kb non-parental fragment when the *Bam*HI-digested DNA was hybridized with the probe. In the first-backcrossed progeny of this cybrid, the hybridization pattern was not stably inherited. A 4.0 kb radish fragment, not detectable in the cybrid, appeared in one of the BC_1 generation siblings, and the 2.0 kb non-parental fragment was lost in another. The hybridization patterns in BC_1 progeny siblings of cybrid 12-9 were also varied. The alteration of mtDNA in the cybrid progeny continued to the BC_2 generation. There was no clear evidence of a heteroplasmic state or of sub-stoichiometric molecules in the mt genome of cybrid 18-3. A possible cause of the observed alteration in the mt genome is discussed.

Key words: *B. napus* – Male sterility – *R. sativus* – Cybrid – mt genome

Introduction

Within the last decade, traits encoded by cytoplasmic genomes, such as cytoplasmic male sterility and atrazine resistance, have been introduced into desirable species of various crop plants by protoplast fusion (see review by Ichikawa et al. 1988). X- or γ-irradiation of the cytoplasmic "donor" cells prior to protoplast fusion can facilitate chromosome elimination from the cells, which results in the formation of cybrids carrying certain combinations of parental cytoplasmic organelles and a "recipient" nucleus.

In such cybrid plants, unique combinations of the cytoplasmic genomes arise from the sorting out of the organelles and/or recombination of the DNAs during the division and vegetative development of the fused cells. Several authors have investigated the fate of mitochondrial (mt) genomes in cybrid plants of *B. napus* (Vedel et al. 1986; Barsby et al. 1987; Morgan and Maliga 1987; Rosén et al. 1988; Jarl et al. 1989; Kao et al. 1991), petunia (Boeshore et al. 1983), tobacco (Kumashiro et al. 1989), carrot (Tanno-Suenaga et al. 1988), and rice (Akagi et al. 1989; Kyozuka et al. 1989; Yang et al. 1989).

The fate of mtDNA in the sexual progeny of the cybrid, however, has been less thoroughly examined. Izhar et al. (1983) demonstrated unstable fertility in petunia somatic hybrids and their backcrossed progeny and suggested that this was the result of a sorting out of the heteroplasmic state present in the cybrids. Aviv and Galun (1987) reported on mtDNA RFLPs in the sexual progeny siblings of the cybrid between *Nicotiana tabacum* and *N. bigelovii*.

In this paper, we report the successful production of male-sterile cybrid plants via intergeneric "donor-recipient" protoplast fusion between a cytoplasmic male-sterile (cms) line of *R. sativus* and *B. napus*, and investigate the fate of their mtDNA in the sexual progeny by DNA-DNA hybridization. An alteration of the mtDNA genome containing the *atpA* gene was found in the progeny siblings. We discuss the possible cause of this alteration.

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Materials and methods

Plant material

A cms line was isolated from a natural population of the domestic radish (*R. sativus*) cultivar Kosena (Y. Ikegaya, personal communication). This line was kindly provided by Y. Ikegaya and was used as a cms donor in our experiments.

The restriction patterns of the Kosena (kos) mtDNA were slightly different from those of the Ogura (ogu) radish cms line (unpublished data).

Protoplast isolation and pre-fusion treatment

About 200 seeds of *B. napus* cv. Westar were surface-sterilized and germinated on MS hormone-free medium in the dark. Using thin blades, 5–6 day-old hypocotyls were cut into 1–2 mm pieces in an enzyme solution containing 2% cellulase RS (Yakult Honsha Co., Ltd.), 0.05% pectriase Y-23 (Seishin Pharmaceutical Co., Ltd.), 0.4 M sucrose, and the inorganic compounds of R medium (J. Shepard, personal communication). After 16–18 h incubation, protoplasts were recovered by centrifugation, and the isolated protoplasts were treated with 10 mM of iodoacetamide (IOA) for 10 min at room temperature after being washed three times with W5 solution (Menczel and Wolfe 1984) just prior to the fusion treatment.

Kosena protoplasts were isolated from the leaves of 3 week-old shoot cultures maintained on MS medium supplemented with 0.1 mg/l NAA. The detailed method for protoplast isolation has been described elsewhere (Sakai and Imamura 1990).

To inhibit colony formation and to stimulate selective elimination of the radish chromosomes from the fusion products, the protoplasts, suspended in W5 solution at a cell density of 5×10^5 cells/ml, were irradiated with 60 Krad of X-rays (1.4 Krad/min).

Fusion treatment

The IOA-treated *B. napus* protoplasts (cytoplasmic recipient) and the X-irradiated protoplasts of *R. sativus* cv. Kosena (cytoplasmic donor) were mixed in a 1:2 ratio to give a total population density of 2×10^6/ml. Three droplets of 100 µl each were pipetted onto the base of a 6 cm Petri dish and 100 µl of a 40% PEG solution (Zellkulter Boehringer Mannheim PEG 1500) was added to each protoplast droplet to give a final concentration of 20% PEG.

Chromosome observations

Chromosome checks were made on the root tips of parental species and fusion products by the method of Nishibayashi and Kaeriyama (1986).

Protoplast isolation and callus formation from the fusion product

Protoplasts were isolated from fully expanded leaves of the acclimated plant 18-3. Enzyme digestion, purification, and culture of the mesophyll protoplasts of cybrid 18-3 were performed using the method described above for *R. sativus* protoplasts.

Isolation of total cellular DNA

Total cellular DNAs were isolated from the leaves or the inflorescence of the parental lines and their fusion parents according to the method described by Fedoroff et al. (1977). One microgram of DNA was digested with *Bam*HI at 37 °C overnight and separated by electrophoresis in an 0.7% agarose gel (Sigma, Type II). The digested total DNA in the gel was denatured in an alkaline solution (0.5 N NaOH, 1.5 M NaCl) for 30 min and transferred from the gel to a nylon membrane filter (Hybond-N, Amersham) with vacuum blotting (Vacugene, LKB).

To identify mtDNA, a cloned pea mtDNA fragment containing the *atpA* gene (Morikami and Nakamura 1987) was used as a probe. This gene was kindly provided by Dr. K. Nakamura.

A 1.7 kb *Bam*HI fragment of the plasmid was electroeluted and labeled with α-^{32}P-dCTP using a random primer labeling kit (Multiprime, Amersham).

The filters were hybridized with the probe at 42 °C for 16 h and successively washed with 2 × SSC, 0.5 × SSC and 0.1 × SSC for 15 min each at 65 °C, followed by exposure to Kodak OMAT-AR films.

Results

Chromosome number and flower morphology of the regenerated plants from "donor-recipient" protoplast fusion

X-irradiated radish mesophyll protoplasts and IOA-treated *B. napus* hypocotyl protoplasts were fused with PEG. The estimated fusion frequency between the hypocotyl protoplast and the mesophyll protoplasts was approximately 10% in each fusion experiment. Control experiments involving self-fusion of *B. napus* protoplasts with IOA treatment and self-fusion of *R. sativus* with X-irradiation were always carried out in parallel and never produced growing colonies. Table 1 shows the chromosome number and flower morphology of ten putative cybrids derived from three independent fusion experiments. These ten fusion products were classified into three types according to their stamenal phenotype.

Type A included four aneuploid plants having flowers characterized by shrunken anthers, containing no functional pollen grains, and normal length filaments (Fig. 1 A, D). These fusion products recovered their male fertility following a single backcross. Type B plants were

Table 1. Chromosome number and flower morphology of the plants derived from "donor-recipient" protoplast fusion between an *R. sativus* Kosena cms line and *B. napus* cv. Westar

Plant no.	Petal color	Chromosome no.	Stamen phenotype[a]	Male fertility
6-5	Yellow	57	B	Sterile
6-8	Yellow	56	C	Fertile
6-14	Yellow + white[b]	84	A	Sterile
6-15	Cream	50	A	Sterile
6-16	Yellow	60	A	Sterile
12-9	Yellow	38	B	Sterile
12-12	Yellow + white[b]	53	A	Sterile
18-3	Yellow + white[b]	38	B	Sterile
18-4	Cream	46	B	Sterile
18-6	Cream	47	B	Sterile

[a] Stamen phenotype of the regenerated plants are classified as: type A, plants which have normal length filaments and shrunken anthers; type B, plants which have stunted filaments and empty anthers; type C, plant which has flowers identical to those of *B. napus*

[b] Yellow petals with white sectors

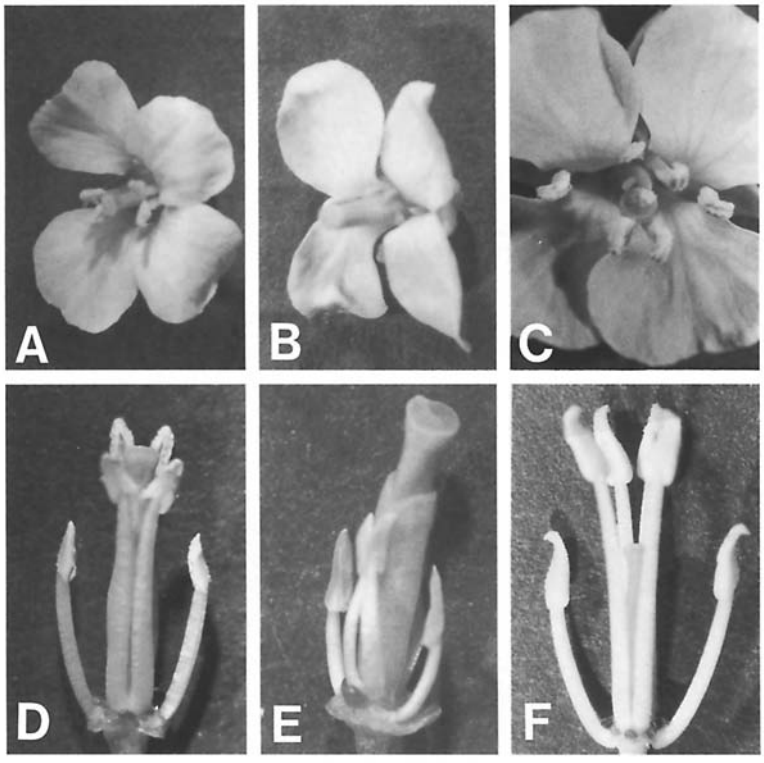

Fig. 1 A–F. Fertile and male-sterile flowers of the regenerated plants from the protoplast fusion between *R. sativus* cms line Kosena and *B. napus* cv. Westar. The plants are classified into three types as described in the Results section. The intact flower is shown on the top row and the flower organs on the bottom. **A** and **D**, a flower of a type A plant (6-16); **B** and **E**, a flower of a type B plant (18-3); **C** and **F**, a flower of a type C plant (6-8)

characterized by stunted filaments with empty anthers irrespective of their chromosome number (Fig. 1 B, E). The male sterility was stably inherited to the BC_1 progeny. One aneuploid plant (6–8) possessed fully developed stamens (Fig. 1 C, F) with functional pollen grains, suggesting that this plant might be a surviving self-fusion products of *B. napus*.

Two diploid fusion products (18-3 and 12-9), which showed stable male sterility and set seeds when backcrossed, were selected for further investigation of the mt genome.

Hybridization pattern of atpA in the BC_1 and BC_2 progeny

To identify mt genomes in parental lines and in their fusion product, 18-3, we isolated total cellular DNA from the mature leaves and digested it with *Bam*HI. ^{32}P-labeled mitochondrial pea *atpA* was used as a hybridization probe against the endonuclease-digested DNAs. No cross hybridization signal was observed when the *atpA* was hybridized with the cpDNA of both parents (data not shown).

The *atpA* hybridized to a 4.0 kb and a 3.0 kb *Bam*HI fragment in *R. sativus* and *B. napus*, respectively. In 18-3, this probe hybridized to both a 3.0 kb fragment, characteristic of *B. napus* mtDNA, and a 2.0 kb non-parental fragment. No hybridization signal was observed at 4.0 kb (Fig. 2 A).

To investigate the fate of mt genomes in the sexual progeny of 18-3 and 12-9, we isolated total DNAs from mature leaves in the first-backcrossed generation (BC_1). The *atpA* gene was again used as a probe against *Bam*HI-digested total DNA of the progeny (Fig. 2 A, B). The siblings in BC_1 were grouped into categories from type 1 to type 4 by the hybridization pattern of their total DNA (Fig. 3).

Type 1 plants (12-9-1, 12-9-3, 12-9-10) had parental 4.0 kb and 3.0 kb fragments characteristic of *R. sativus* and *B. napus*, respectively. Type 2 plants (18-3-1, 12-9-5, 12-9-6, 12-9-7, 12-9-8) had both the parent-specific 4.0 kb and 3.0 kb fragments and an additional non-parental 2.0 kb fragment. Type 3 plants (18-3-2, 18-3-6, 18-3-7, 18-3-8, 12-9-2) had a 3.0 kb fragment characteristic of *B. napus* and the novel 2.0 kb fragment. A type 4 plant (18-3-9) had only the 3.0 kb fragment characteristic of *B. napus* (Figs. 2 A, B and 3). These data demonstrate that the hybridization pattern in 18-3 and 12-9 was not stably inherited by BC_1 and segregated in the siblings of BC_1 progeny. All of the BC_1 progeny analyzed contained the 3.0 kb fragment specific to *B. napus*.

We further investigated the mtDNAs in the cybrids and their second backcrossed generation (BC_2). Type 2 plants 18-3-1 and 12-9-5 were pollinated with pollen grains of *B. napus* cv. Westar, and mitochondrial genomes of the siblings in BC_2 were analyzed by hybridization using *atpA* as a probe (Fig. 4).

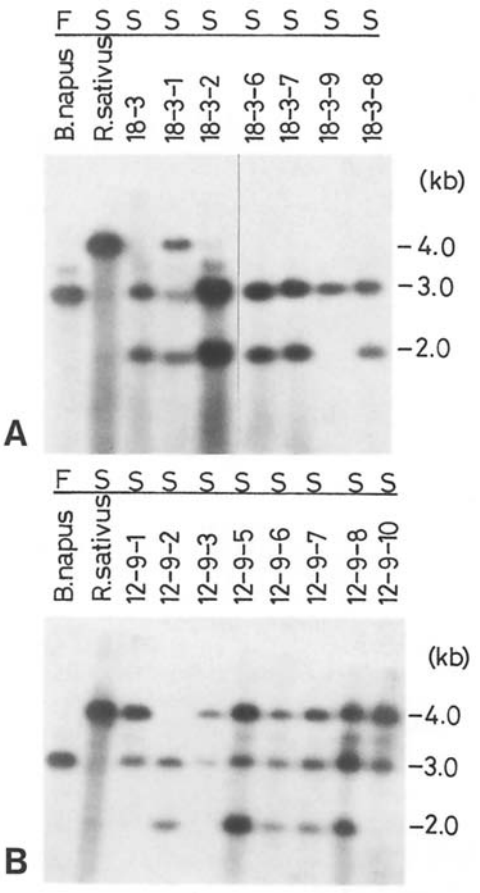

Fig. 2 A, B. Southern hybridization analysis of total cellular DNA of cybrids and their first backcrossed progeny (BC_1). *Bam*HI-digested total cellular DNA was separated by 0.7% agarose, transferred to a nylon membrane and hybridized with ^{32}P-labeled *atpA* as a probe. **A** *B. napus* cv. Westar (*lane 1*); *R. sativus* cms line, Kosena (*lane 2*); cybrid 18-3 (*lane 3*), and BC_1 progeny (*lanes 4–9*). **B** *B. napus* cv. Westar (*lane 1*); *R. sativus* cms line Kosena (*lane 2*), and BC_1 progeny of cybrid 12-9 (*lanes 3–10*). *F* and *S* indicate male-fertile and male-sterile flowers, respectively, of the fusion parent, cybrids and the progeny. About 1 µg of total cellular DNA was loaded on each lane. Fragment sizes are given in kb

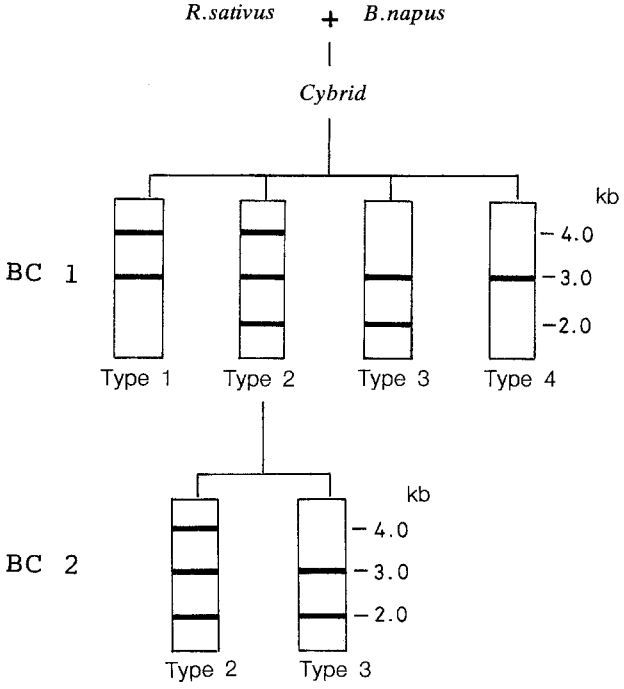

Fig. 3. Schematic representation of the classified hybridization patterns in BC_1 and BC_2 progeny of a *R. sativus* + *B. napus* cybrid. The plants were grouped into four categories by the hybridization pattern of their total DNA

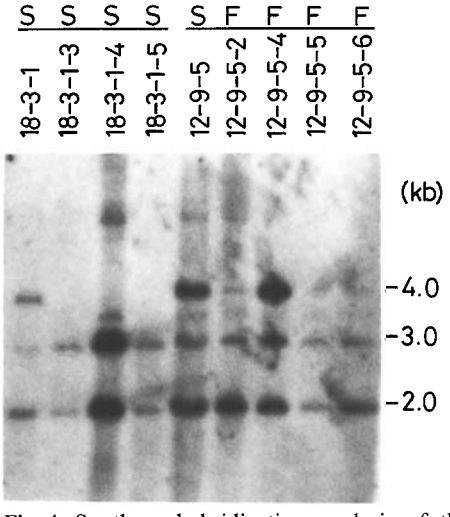

Fig. 4. Southern hybridization analysis of the second backcrossed (BC_2) progeny and their progenitors. *Bam*HI-digested total cellular DNA was hybridized with ^{32}P-labeled *atpA* as a probe. Plant 18-3-1 (*lane 1*) and 12-9-5 (*lane 5*) are the progenitors of the BC_2 progeny. *Lanes 2–4* and *6–9* are BC_2 progeny. *F* and *S* indicate the male-fertile and male-sterile flowers, respectively, of the cybrid progeny. Fragment sizes are given in kb

All siblings in the BC_2 progeny of 18-3-1 had the 3.0 kb fragment specific to *B. napus* and the 2.0 kb non-parental fragment. The 4.0 kb fragment found in 18-3-1 was, however, absent (Fig. 4). Two out of four siblings (12-9-5-2 and 12-9-5-4) in the progeny of 12-9-5 showed identical hybridization to that of the female parent 12-9-5. A sub-stoichiometric difference in the 4.0 kb fragment was observed in 12-9-5-2 (Fig. 4). Two other siblings, 12-9-5-5 and 12-9-5-6, had no 4.0 kb fragment. These data indicated that the hybridization patterns in both BC_1 lines 12-9-5 and 18-3-1 were not inherited stably by BC_2.

To determine whether or not the observed variations of the hybridization patterns among the progeny in BC_1 and BC_2 were due to heteroplasmic mitochondrial genomes we compared hybridization patterns between different tissues in a plant or different cells in a tissue.

Total DNA was isolated from the inflorescence in different branches, or from the calli derived from mesophyll protoplasts, of 18-3 and was digested with *Bam*HI.

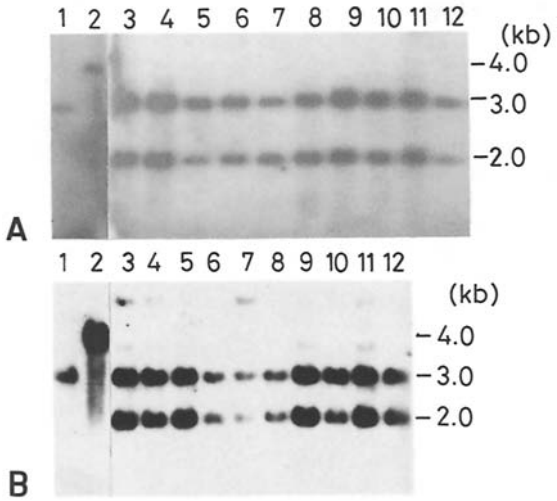

Fig. 5 A, B. Southern hybridization analysis of *Bam*HI-digested total cellular DNA of the different inflorescences of calli derived from the cybrid 18-3 using *atpA* as a probe. **A** *B. napus* (*lane 1*), *R. sativus* (*lane 2*) and ten different inflorescences of cybrid 18-3 (*lanes 3–12*). **B** *B. napus* (*lane 1*), *R. sativus* (*lane 2*) and ten cloned calli derived from mesophyll protoplasts of cybrid 18-3 (*lanes 3–12*). Fragment sizes are given in kb

The DNA was hybridized with *atpA* as a probe and the hybridization patterns were compared.

The hybridization patterns among ten different inflorescences of 18-3 were identical to each other (Fig. 5A) and to those of the cloned calli derived from the mesophyll protoplast (Fig. 5B). This suggests that the mitochondrial genomes of 18-3 are homoplasmic throughout the plant rather than "chimeric" in each tissue or organ.

Male fertility in the progeny of cybrids 12-9 and 18-3 was investigated. All of the BC_1 and BC_2 siblings of 18-3 showed male sterility (Figs. 2A, 4). Although all BC_1 siblings of 12-9 were male-sterile, male fertility segregated in the BC_2 generation where three fertile revertants appeared (Fig. 4). The percentage of mature pollen grains in the dehisced anthers of these fertile revertants ranged from 20 to 40%.

Discussion

Intergeneric cms transfer by "donor-recipient" protoplast fusion

Several authors have reported the transfer of Ogura radish cms to *B. napus* by symmetric or asymmetric protoplast fusions (Pelletier et al. 1983; Menczel et al. 1987; Morgan and Maliga 1987; Rosén et al. 1988; Jarl et al. 1989; Kao et al. 1991). In these cases, the radish cytoplasm could be transferred between cultivars of *B. napus* because the cytoplasm had been introduced into *B. napus* by conventional backcrossing. However, Kosena radish cms has not been introduced into any *Brassica* species. Therefore, "donor-recipient" protoplast fusion provides a useful tool to directly introduce this cytoplasm through the natural barriers existing in *B. napus* and *R. sativus*.

In our experiments, most of the regenerated plants showed male sterility. However, all of the type A plants reverted to their original fertility in the BC_1 generation. In addition, some of the chromosomes were eliminated in the BC_1 plants, suggesting that the observed temporal male sterility found in type A plants was caused by impairment of the parental chromosomes. Chromosome impairment has previously been shown to affect the fertility of interspecific hybrids in *B. napus* (Namai et al. 1980). All of the type A plants, except 6-16, possessed cream-colored flowers or yellow-colored flowers with white sectors. White-colored radish flowers is known to be a dominant character in *Raphanobrassica*. Therefore, radish chromosomes retained in the type A plant may cause the temporal male sterility.

mtDNA alteration in the progeny of the cybrids

We find that a unique mitochondrial genome caused by a heteroplasmic protoplast fusion is not stably inherited in the sexual progeny of a cybrid. Alterations of mitochondrial genomes in the siblings of sexual progeny have been reported in somatic hybrids of tobacco (Aviv and Galun 1987) and petunia (Izhar et al. 1983). Thus the observed instability of mtDNA among progeny siblings might be a rather general phenomenon in the progeny of a somatic hybrid or a cybrid. In our preliminary experiments, alteration of the mtDNA in BC_1 and BC_2 progeny was also observed when *coxII, atp6,* and *26Srrn* were used as probes, indicating that the observed alterations of the mt genome occurred not only in *atpA*, or its flanking region, but also over a wide range of the genome.

Segregation of the *B. napus* cybrid mtDNA is completed during an early stage of callus development (Morgan and Maliga 1987). Southern hybridization of inflorescences and calli derived from mesophyll protoplasts of 18-3 indicated that mtDNA in this cybrid might be uniform rather than chimeric (Fig. 5A, B).

In contrast to the situation in the cybrid, however, the hybridization patterns of the mtDNA differed in the progeny. A 4.0 kb fragment not detectable in cybrid 18-3 appeared in one of the progeny and disappeared again in the next generation (Figs. 2A, 4). Alterations in mtDNA were also observed in other lines of the progeny. In all lines examined, the *atpA* gene hybridized predominantly with three definite fragments – 4.0 kb, 3.0 kb, and 2.0 kb. No other hybridization was detected. These observations strongly imply that mtDNA alterations among progeny siblings were due to large differences in the stoichiometry of the existing mt genome constitution rather than to de novo recombination. Small et al. (1987, 1989) reported that the copy number of mtDNA molecules containing

atpA retained at low abundance ("sublimons") alters its stoichiometry through many generations, while Wang and Gengenbach (1989) reported that the *atpA* in maize S cytoplasm changes its stoichiometry over several generations. This rapid change is most likely due to differences in the stoichiometry of existing molecules (Escote-Carlson et al. 1990). Similarly, the appearance of novel mtDNA molecules in cultured cells of *B. campestris* could be due to an amplifications of pre-existing low-abundance molecules (Shirzadegan et al. 1989, 1991). Although we would expect the existence of sub-stoichiometric molecules in cybrids and their progeny, there is no obvious evidence of this so far. Further studies are required to explain the observed alterations of mtDNA in the cybrid progeny.

Plant 18-3-9, which only possessed the 3.0 kb fragment characteristic of *B. napus*, showed male sterility. Plant 12-9-5-2, with type 2 mtDNA, reverted to fertility, whereas other siblings with the same type of mtDNA showed male sterility. These results indicate that Kosena *atpA* is not associated with male sterility of the cytoplasm. Likewise, Makaroff et al. (1990) reported that transcripts of the Ogura radish *atpA* are not involved in male sterility.

In BC_2 siblings of 12-9, fertile revertants were observed. Instability of fertility has also been reported in the progeny of a *B. napus* cybrid carrying Ogura cms cytoplasm (Bonhomme et al. 1991). We are investigating whether this instability is correlated with the wide range of alterations in the mt genome observed in this study.

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