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Intergeneric transfer of cytoplasmic male sterility between *Raphanus sativus* (cms line) and *Brassica napus* through cytoplast-protoplast fusion

T. Sakai* and J. Imamura

Plantech Research Institute, 1000 Kamoshida, Midori-ku, Yokohama 227, Japan

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Summary. Cytoplasts isolated from hypocotyl protoplasts of *Raphanus sativus* cv Kosena (cms line) by ultracentrifugation through Percoll/mannitol discontinuous gradient were fused with iodoacetamide(IOA)-treated protoplasts of *Brassica napus* cv Westar. Seventeen randomly selected regenerated plants were characterized for morphology and chromosome numbers. All of the regenerated plants had morphology identical to *B. napus* and 10 of them possessed the diploid chromosome number of *B. napus*. The remaining plants had chimeric or aneuploid chromosome numbers. The mitochondrial genomes in the 10 fusion products possessing the diploid chromosome numbers of *B. napus* were examined by Southern hybridization analysis. Four of the 10 plants contained mitochondrial DNA showing novel hybridization patterns. Of these 4 plants, 1 was male sterile, and 3 were male fertile. The remaining plants showed mitochondrial DNA patterns identical to *B. napus* and were male fertile.

Key words: *B. napus* – Cytoplast – Fusion – Cybrid – cms transfer

Introduction

In somatic hybrid cells, formation of unique cytoplasms that differed from those of the parents have been observed (see review, Ichikawa et al. 1988). Random sorting-out of chloroplasts and recombination of mitochondrial and/or chloroplast DNAs might cause the formation of unique cytoplasms in the somatic hybrid cells (Izhar et al. 1983; Pelletier et al. 1983; Menczel et al. 1986; Kumashiro et al. 1989). Somatic hybridization thus provides a method for combining desired traits encoded on the organelles of the parental species.

In somatic cell hybridization, X- or γ-ray irradiation to cytoplasmic "donor" cells prior to cell fusion treatments stimulates selective elimination of the chromosomes (see review, Hinnisdeals et al. 1988). Thus, the repeated backcrossing required for transfer of cytoplasmic traits in conventional breeding can be avoided.

Transfer of chloroplast and mitochondria is now routinely done by "donor-recipient" protoplast fusion in several crop plants (Barsby et al. 1987; Menczel et al. 1987; Akagi et al. 1989; Yang et al. 1989; Kyozuka et al. 1989). In oilseed rape, the cytoplasmic traits that could be of value for breeding, such as cytoplasmic male sterility and atrazine resistance, were transferred by somatic hybridization (Pelletier et al. 1983; Barsby et al. 1987; Jourdan et al. 1989a, b). However, in *Brassica* species, the effects of irradiation on the elimination of chromosomes are limited, so that some chromosomes of the "donor" cells might be retained (Menczel et al. 1987; Barsby et al. 1987). An alternative method for the transfer of organelles is based on fusion between enucleated cells (cytoplasts) and nucleated cells (karyoplasts or protoplasts) (Wallin et al. 1979).

Maliga et al. (1983) demonstrated that tobacco chloroplasts bearing streptomycin resistance could be transferred by cytoplast-protoplast fusion.

In order to examine the possibility of producing cybrids and transferring traits encoded in organellar genomes by cytoplast-protoplast fusion in *Brassicaceae*, a cms line of *Raphanus sativus* and *B. napus* was used as cytoplasmic donor and recipient, respectively. Through cytoplast-protoplast fusion we obtained cybrid plants carrying rearranged mitochondrial DNAs, with one of them showing male sterility.

* To whom correspondence should be addressed

Materials and methods

Plant material

The cms line of Kosena, found and isolated by. Ikegaya (Ishii seed, 710 Ikeda, Shizuoka, Japan 422) from a population of a domestic cultivar of radish (*Raphanus sativus* cv Kosena), was used for cytoplast preparation.

Protoplast and cytoplast isolation

About 200 radish seeds were used in each cytoplast isolation experiment, after being surface-sterilized with 5% NaClO for 20 min and rinsed several times with sterile water. The sterilized seeds were germinated in the dark on MS medium without hormones at 27 °C. Five- to seven-day-old hypocotyls were sliced 1 mm thick in plasmolysis solution containing 0.3 *M* sorbitol, 0.05 *M* $CaCl_2$, pH 5.6 (Glimelius 1984). The plasmolysis solution was then replaced by an enzyme solution containing 2% Cellulase-RS, 0.5% Macerozyme-R10 (Yakult Honsha Co.), 0.05% Pectolyase Y-23 (Seishin Pharmaceutical Co.), 0.35 *M* sucrose, and NN67 (Nitsch and Nitsch 1967) inorganic components. After overnight incubation at 27 °C, the suspension was sieved through 250 mesh nylon net to remove undigested debris and, finally, the protoplasts were washed twice with W5 solution (Menczel and Wolfe 1984).

Cytoplasts were isolated from the *R. sativus* protoplast preparation by Percoll/mannitol density gradient ultracentrifugation, which was a modification of the method described in Lörz et al. (1981). One milliliter of each of three iso-osmotic solutions of different density was overlayered in a 5-ml polyallomer tube (from bottom to top: 50% Percoll diluted with 0.9 *M* mannitol; 20% Percoll diluted with 0.6 *M* mannitol; 0.6 *M* mannitol); then 1 ml of protoplast suspension containing 10^6 cells in W5 solution was layered on top of the gradient. Centrifugation was done at 12 °C in a Hitachi SPH-70 ultracentrifuge equipped with a RPS55T-2 swing bucket rotor. All the cytoplast preparations were manipulated under aseptic conditions. After ultracentrifugation, the cytoplasts were recovered with a Pasteur pipette, then diluted with 5 vol. of W5 solution, and carefully washed with W5 solution.

Protoplasts were isolated from leaves of shoot cultures of *B. napus* cv Westar that had been maintained at 25 °C on MS basal medium (Murashige and Skoog 1962) containing 3% sucrose, 0.1 mg/l NAA and 0.6% agarose. The leaves were excised from 3-week-old shoots, but into 1–2 mm pieces, and macerated overnight in enzyme solution containing 0.4 *M* sucrose, NN67 inorganic components, 0.5 mg/l 2,4-D, 0.5 mg/l NAA, 1 mg/l BAP, 2% Cellulase-RS, 0.5% Cellulase R-10, 0.5% Macerozyme R-10, and 0.02% Pectolyase Y-23. Protoplasts were recovered by sucrose flotation after 16–18 h of incubation in the enzyme soluation. These isolated protoplasts were treated with 7 m*M* iodoacetamide at room temperature for 7 min, then washed three times with W5 solution just prior to fusion treatment.

Fusion treatment

Cytoplasts and protoplasts were suspended separately in W5 solution at a density of 2×10^6 cells/ml and mixed in a 2:1 ratio, after which three drops (100 µl each) were placed on 60-mm petri dishes and kept for 5 min to sediment the cells. Fifty microliters of 40% PEG (Boehringer Mannheim, 50% PEG 1,500) diluted with W5 solution was then gently added dropwise and kept in the dish for 10 min. After removal of the liquid from the dish with a Pasteur pipette, the cells were washed carefully by adding 100 µl of 6.7% PEG solution, then allowed to settle for 10 min (Sundberg and Glimelius 1986). Finally, the PEG solution was carefully removed, and 3 ml of KM8p medium containing 0.4 *M* glucose, 1 mg/l 2,4-D, 0.1 mg/l NAA, 0.4 mg/l BAP (Glimelius 1984) was added to each petri dish.

Plant regeneration

When the fused cells began to divide, 0.5 ml of KM8p medium containing 0.1 *M* sucrose instead of glucose was added to each petri dish to dilute the culture medium. This dilution medium (0.5 ml) was added to each dish weekly (Glimelius 1984). Small colonies (0.5–1 mm in diameter) that were present after 4 weeks of culture were transferred to proliferation medium (Barsby et al. 1987). Calli (3–5 mm in diameter) that formed on the proliferation medium were picked up with forceps and transferred to 0.5% agarose-solidified MS basal medium containing 0.2 *M* mannitol, 6 m*M* sucrose, 100 mg/l casein hydrolysate, 80 mg/l adenine sulfate, 0.1 mg/l IAA, 2 mg/l kinetin, and 2 mg/l zeatin to induce shoot formation (J. Shepard, personal communication). The plantlets that regenerated were cultured on MS medium containing 3% sucrose and 0.1 mg/l NAA. Morphologically normal plants were transferred to vermiculite for acclimation.

Microscopical test for enucleation

After fractionating, recovered cells were suspended in W5 solution and fixed for 20 min at room temperature with glutaraldehyde at the final concentration of 0.25% (v/v), to determine the frequency of enucleated cells. After fixing, the cells were washed gently with W5 solution. Samples (50 µl) of the fixed cell suspensions were stained on slide glasses by adding 10 µl of 1 mg/l DAPI dissolved in water. Cells were examined under epifluorescent illumination on a Nikon Optiphoto XF-EFD equipped with a UV excitation filter (Nikon filter UV). At least 200 cells were observed when determining the frequency of enucleation.

Chromosome analysis

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

Identification of mitochondrial DNA by Southern blot analysis

Total cellular DNA was isolated from leaves of the parental species and segregated fusion products, according to Fedoroff et al. (1977), and digested with BamHI. The DNA fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to a nylon membrane (Amersham, Hybond-N) with Vacugene (LKB). Four cloned mtDNA fragments that contained genes for pea cytochrome oxidase subunit II (*coxII;* A. Morikami and K. Nakamura, unpublished data), *Oenothera* cytochrome oxidase subunit III (*coxIII;* Hiesel et al. 1987), pea 18S ribosomal RNA (*rrn18;* A. Morikami and K. Nakamura, unpublished data), and pea ATP-synthase subunit A+9 (*atpA+9*; Morikami and Nakamura 1987) were used as hybridization probes. *coxII, atpA+9,* and *rrn18* were kindly provided by K. Nakamura, (Nagoya University, Japan) and *coxIII* by D. Lonsdale (Plant Breeding Institute, Cambridge, UK). Membranes were hybridized with random-primed ^{32}P-labeled mtDNA fragments (Multiprime Labeling Kit, Amersham) following the manufacturer's recommendations, and washed successively with $2 \times$ SSC, $0.5 \times$ SSC, and $0.1 \times$ SSC solution at 65 °C for 15 min each washing, then exposed to Kodak OMAT-AR film at −80 °C with intensifying screens for 16–20 h.

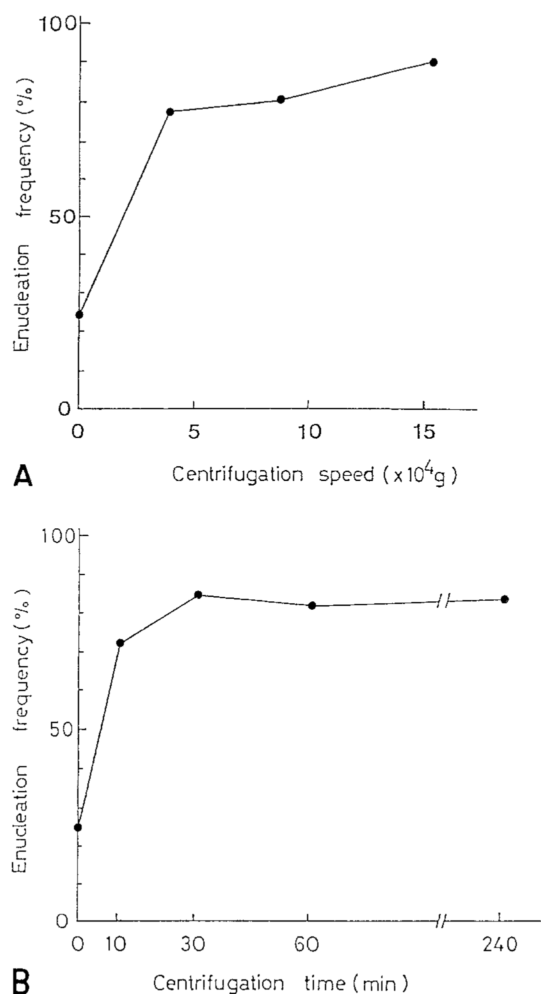

Fig. 1 A and B. Enucleation frequency of cells recovered from the interphase between the 0.6 *M* mannitol and W5 layers. Effects of different centrifugation speeds (**A**) and different periods of centrifugation (**B**) on the enucleation frequency. Centrifugation was done at 12 °C for 60 min and 38,000 × g if not otherwise stated

Results

Cytoplast isolation

Parameters that might affect the cytoplast yield from hypocotyl protoplasts of *Raphanus sativus* cv Kosena were tested. Yields were not significantly affected by temperatures from 8 °C to 25 °C during preparation procedures (data not shown). Enucleated cells appeared during incubation for protoplast isolation with a frequency of 10%–30% before fractionation by ultracentrifugation. The speed and duration of centrifugation affected the frequency of the enucleated cells in the cytoplast fraction. The cytoplast yield increased with increasing gravity from 38,000 to 152,000 × g (Fig. 1 A). Most cytoplasts were isolated from the protoplasts during the first 30 min of centrifugation; further centrifugation had no effect on cytoplast yield (Fig. 1 B). Therefore, cytoplasts were routinely isolated by centrifugation at 12 °C, 40,000 rpm (152,000 × g) for 30 min.

After centrifugation two bands, one at the interphase between 50% and 20% Percoll and one at the interphase between the 0.6 *M* mannitol and W5 layer, were observed. These bands were recovered and cells were stained with the fluorescent dye DAPI to establish the frequency of enucleation. Figure 2 shows the same field of DAPI-stained cells from the upper gradient fraction under normal light and epifluorescent illumination. Intact cells with large vacuoles were predominantly observed in this fraction (Fig. 2 A). Nonenucleated cells with brightly stained nuclei were identified under epifluorescent illumination (Fig. 2 B, indicated by arrows). Of the recovered cells, 80%–95% were enucleated. Reproducibly ca. 5×10^5 cytoplasts could be recovered from 10^6 protoplasts, which proved to be sufficient for cytoplast-protoplast fusion experiments.

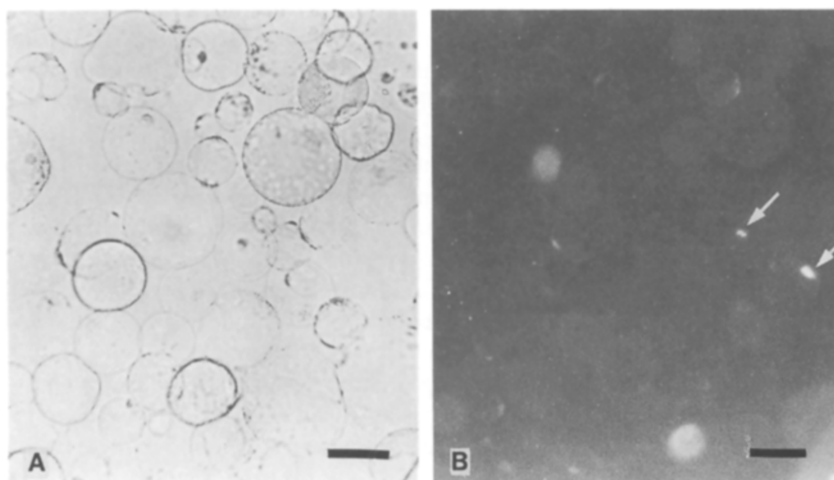

Fig. 2 A and B. Microscopic observation of the cytoplast fraction recovered from interphase between 0.6 *M* mannitol and W 5 stained with DAPI. **A** brightfield and **B** fluorescence optics of the same field showing cytoplasts, *Arrows* indicate nuclei of protoplasts in cytoplast fraction. Bars represent 80 μm

Fig. 3. A cybrid plant (no. 14) showing male sterility derived from the cell fusion of *Raphanus sativus* cytoplast with B. napus protoplast

Cell fusion and plant regeneration

Immediately after isolation, cytoplasts of *R. sativus* were fused with IOA-treated protoplasts of *B. napus*. The number of fusion products was significantly affected by the cytoplast-to-protoplast ratio in the cell mixture used for fusion. No colonies were recovered when cytoplasts and protoplasts were mixed in equal proportions before the fusion treatment.

Cell division began 7–10 days after the fusion treatment. When compared to protoplast-protoplast fusion for *R. sativus* and *B. napus*, the onset of cell division was delayed 2–5 days. Once cell division began, the cells proliferated at a rate similar to those of cells produced by protoplast-protoplast fusion. The plating efficiency of the cytoplast-protoplast fusion products was approximately 0.01%. Fusion products were grown and regenerated to plants by the method used to regenerate *B. napus* protoplasts (see "Materials and methods"). Regeneration frequency was the same as for *B. napus* cv Westar protoplast culture (5%–10%). Out of five fusion experiments done, colonies and shoots were recovered from two independent experiments. Control experiments involving self-fusion of IOA-treated *B. napus* protoplasts and the culture of *R. sativus* cytoplasts with or without PEG treatment, which were run in parallel, never produced growing colonies.

The chromosome number and morphology of regenerated plants

The ploidy level was estimated in 17 randomly selected regenerated plants before their transfer to the greenhouse. Of these 17 plants, 10 had 2n = 38 chromosomes; 5 had chimeric tissue with cells showing diploid (2n = 38) and aneuploid (2n = 40–60); and 2 had 2n = 47 to 50 chromosomes. None of the regenerated plants tested had the chromosome number of *R. sativus* (2n = 18); all showed shoot and leaf morphology identical to those of *B. napus* (Fig. 3). Plants that had 2n = 38 chromosomes may represent regenerants from fusion products of cytoplasts and protoplasts, from "escaped" cells of *B. napus* or nuclear segregants after protoplast-protoplast fusion, due to contaminating *Raphanus sativus* protoplasts in the cytoplast fraction. Among the 10 regenerated plants that had *B. napus* chromosome number (2n = 38), 9 showed male fertility with well-developed stamens and anthers (Fig. 4A, B), and 1 plant (no. 14) was male sterile with stunted filaments and aborted anthers (Fig. 4C, D). The petal shape and color of these regenerants were identical to those of *B. napus*. The male-sterile regenerant set seeds when backcrossed with pollen of *B. napus* cv Westar. Its progeny was male sterile.

Southern blot analysis of total cellular DNA with mitochondrial genes

To investigate the mitochondrial genomes of the 10 regenerated plants that had the diploid chromosome number of *B. napus*, five heterologous mitochondrial genes were used as probes and hybridized to BamHI-digested total cellular DNA of the fusion products and their parents.

Figure 5 shows the hybridization patterns of four fusion products (lanes 1 to 4) and their parents, *B. napus* cv Westar (lane 5) and *Raphanus sativus* cv Kosena (lane 6). The cytoplasms of both parental species could not be distinguished by hybridization with mt genes *coxIII* (Fig. 5D). When probed with *rrn18*, fusion products no. 14 (lane 1), no. 5 (lane 3),and no. 1 (lane 4) showed hybridization patterns identical to *B. napus*. Fusion product no. 6 (lane 2) showed the presence of a novel 9.8-kb fragment (Fig. 5A). When probed with *atpA* + *9*, *coxII*, or *coxIII*, all the fusion products tested except plant no. 6 contained a novel 5.6-kb, 9.4-kb, and 14.0-kb hybridization fragment, respectively (Fig. 5B, C, D). No cross-hybridization was observed between all the mt gene probes used and parental cpDNAs, except for *rrn18* (data not shown). A cloned fragment of *rrn18* hybridized with a 3.2-kb parental cpDNA band (Fig. 5A, indicated by arrow).

The remaining 6 plants tested showed the same hybridization patterns as *B. napus*. No evidence for mitochondrial DNA rearrangement was found in these fusion products by Southern hybridization analysis.

Fig. 4 A–D. Fertile and male-sterile flowers of the fusants of cytoplast-protoplast fusion. **A** Fertile flower of a fusant no. 6. **B** Flower organs of no. 6. **C** Male-sterile flower of no. 14. **D** Flower organs of no. 14. The *arrow* indicates an abortive anther with stunted filament

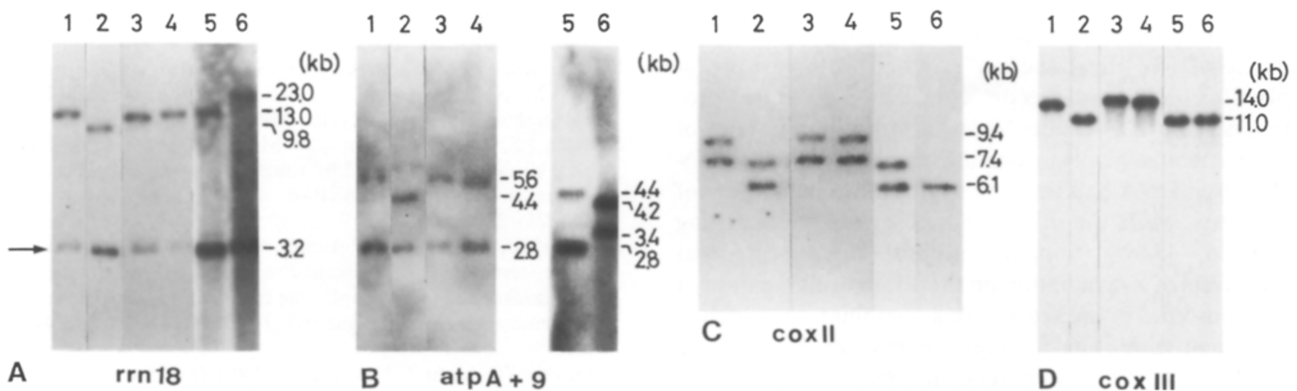

Fig. 5. Southern blot analysis of total cellular DNA from fusants and their parents. Total cellular DNA was isolated from the leaves and digested with BamHI, electrophoresed in 0.7% agarose gels, transferred to nylon membranes, and hybridized with the four cloned fragments containing five heterologous mtDNA genes. The probes used for hybridization are described under each figure *Lane 1*: fusant no. 14, *lane 2*: no. 6, *lane 3*: no. 5, *lane 4*: no. 1, *lane 5*: *B. napus* cv Westar, and *lane 6*: *Raphanus sativus* cv Kosena cms line. Fragment sizes are given in kb. The *arrow* indicates a chloroplast DNA fragment that hybridizes to probe *rrn18*.

Discussion

The results presented here show that cytoplast-protoplast fusion of *Raphanus sativus* (cms line) and *B. napus* results in fertile and sterile cybrids. Introduction of Ogura radish cms cytoplasm to *B. napus* was first carried out by recurrent backcross through *B. oleracea* as a bridge plant (Bannerot et al. 1974), and the resulting *B. napus* carrying the radish cytoplasm has been used as a material for somatic cell fusions to transfer the cytoplasm into fertile varieties of *B. napus* (Pelletier et al. 1983; Menczel et al. 1987; Jourdan et al. 1989a, b). The cytoplast-protoplast fusion presented here permitted the transfer of a newly found CMS cytoplasm in *Raphanus sativus* cv Kosena to *B. napus* cv Westar in a single step.

In a wide range of plant species, extensive alterations of mitochondrial DNA organization can be promoted by tissue culture manipulations (Dale et al. 1981; Negruk et al. 1986; Rode et al. 1987; Brears et al. 1989; Hartmann et al. 1989; Shirzadegan et al. 1989). However, in our protoplast culture, so far no unique hybridization fragments can be observed in total cellular DNA from protoplast-derived plants of *B. napus* cv Westar after Southern hybridization analysis with mitochondrial probes (unpublished data). When radish and oilseed rape cytoplasms were combined through somatic cell fusions, occurrence of novel mitochondrial DNAs have been reported (Chetrit et al. 1985; Menczel et al. 1987; Morgan and Maliga 1987). These facts suggest that the novel hybridization fragments observed in our experiments

might be caused by intermolecular mtDNA recombination and may not represent tissue-culture-induced changes.

Of 17 fusion products tested, 7 had chimeric or aneuploid chromosome numbers and shoot and leaf morphology identical to *B. napus*. This indicates that these plants might not be hybrid plants but rather homoplasmic fusion products of *B. napus* or nuclear segregants of the cytoplast-protoplast fusion products.

Maliga et al. (1983) reported that in cytoplast-protoplast fusion of tobacco, the low frequency of contaminating protoplasts (2%–7%) in the cytoplast preparation may enhance hybrid formation, resulting in a large number of hybrids being formed in the regenerated plants. We, however, found no hybrid plants even when there were large numbers of contaminating *R. sativus* protoplasts (5%–20%) in the cytoplast fraction, which may be due to the low response of *R. sativus* protoplasts in culture.

Both X-or γ-irradiations have been used effectively in a wide range of fusion combinations in "donor-recipient" cell fusion to eliminate nuclear participation of "donor cells" (see "Introduction"). In our protoplast-protoplast fusion experiments on X-irradiated *R. sativus* protoplasts and IOA-treated *B. napus* protoplasts, 2 out of 18 regenerated plants possessing rearranged type of mitochondrial DNA had the diploid chromosome number of *B. napus*, while the others showed aneuploidy ranging from $2n = 43$ to 84 (unpublished data). This indicates that the effects of X-irradiation on the selective elimination of chromosomes is limited for this combination, which is consistent with other findings reported for *Brassicaceae* (Barsby et al. 1987; Menczel et al. 1987). Cytoplast-protoplast fusion, therefore, should eliminate the repeated backcrossing required for transfer of cytoplasmic factors even after "donor-recipient" cell fusion for some combinations of *Brassicaceae*.

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