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Production and characterization of somatic hybrids between the Japanese radish and cauliflower

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Summary. Somatic hybrids between the Japanese radish and cauliflower (*Brassica oleracea*) were produced by protoplast electrofusion in order to introduce clubroot disease resistance in the Japanese radish (*Raphanus sativus*) into *Brassica* crops. After electrofusion of iodoacetamide-treated cauliflower protoplasts with untreated radish ones, culture was performed under conditions, that allowed only cauliflower protoplasts to regenerate. Out of 40 regenerated plants, 37 were morphologically of a hybrid type and 3 of a cauliflower type. On the basis of isozyme and RFLP analysis, all of the hybrid-type plants tested proved to be true hybrids. Of the 10 true hybrids tested, 9 were found to contain chloroplasts similar to those found in the Japanese radish, while only 1 contained those of the cauliflower. Using two mitochondrial genes as probes, we were able to show that 3 hybrids contained mitochondria of the Japanese radish, with some modification, while 7 hybrids had either parental or new patterns. All of the hybrid-type plants showed resistance to clubroot disease as high as that found in the Japanese radish. Some hybrids were self-fertile. All of the self-fertile hybrids were found to contain 36 chromosomes, indicating that they were amphidiploids. In addition, a few seeds were obtained from a backcross of the self-fertile hybrids to both parents.

Key words: Somatic hybrids – *Raphanus sativus* – *Brassica oleracea* – Clubroot disease – Disease resistance

Introduction

Clubroot disease is one of the most serious diseases affecting *Brassica* crops. It is a root infection that is caused by the fungus *Plasmodiophora brassicae*. Once the roots of the host plant are infected, they swell up like a club resulting in loss of function. Breeding for resistant cultivars has been attempted, but has only been partially successful: after only a few years of cultivation in an infected field, resistant cultivars become susceptible to the disease. This is because there is no genetic source for perfect resistance among species that can be used for cross-breeding with *Brassica*. However, clubroot disease does not cause serious losses in Japanese radish (*Raphanus sativus*) cultivation. Yoshikawa et al. (1979) examined 60 cultivars of Japanese radish for their resistance to the disease and found that 18 did not become infected at all. In order to introduce this resistance into *Brassica* crops, we produced somatic hybrids between the Japanese radish and the cauliflower as bridge plants.

Sexual hybrids between *Raphanus sativus* and *Brassica oleracea* (*Raphanobrassica*) have been produced by several researchers for scientific study purposes or for the production of new forage plants (McNaughton 1973). However, there are some drawbacks to sexual hybridization. First, in a combination of *R. sativus* and *B. oleracea*, hybrids can only be obtained when *R. sativus* is used as the female parent. A reciprocal cross is not possible. As a result, the cytoplasm of *B. oleracea* cannot be introduced into the hybrids. In contrast, mixed cytoplasm can be generated by somatic hybridization. Second, it has been reported that the fertility of *Raphanobrassica* is very low (McNaughton 1973) and that a backcross to *B. oleracea* is very difficult, (though backcross to radish is possible) (Namai et al. 1980). Because somatic hybrids might be somewhat different from sexual hybrids, it was expected that somatic hybrids backcrossable to *B. oleracea* would be obtained. Due to the above reasons we adopted somatic hybridization.

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Materials and methods

Plant material

Japanese radish, *Raphanus sativus* cv 'Sofutori-Miyashige', which is immune to clubroot disease, and cauliflower, *Brassica oleracea* cv 'Nozaki-Wase', which is susceptible, were used as fusion partners.

Protoplast isolation

Hypocotyls of etiolated aseptic seedlings were cut transversely into pieces of 5–10 mm long, with each piece cut longitudinally. They were treated with an enzyme solution containing 1% Cellulase Onozuka R10 (Yakult Honsha Co, Japan), 0.25% Macerozyme R10 (Yakult Honsha Co, Japan). 5 m*M* $CaCl_2$ and 0.5 *M* mannitol (pH = 5.8) overnight at 25 °C. The isolated protoplasts were filtered through six layers of gauze and collected by centrifugation (100 *g*, 2 min). They were washed 3 times with a wash solution containing 0.25 *M* KCl and 5 m*M* $CaCl_2$. Cauliflower protoplasts were treated with an iodoacetamide (IOA) solution containing 5 m*M* IOA and 0.25 *M* KCl for 6 min, then washed. In some experiments we did not carry out this IOA treatment. Protoplasts of each species were suspended in a fusion buffer containing 0.5 *M* erythritol and 5 m*M* $CaCl_2$ to a final density of 5×10^5 protoplasts per milliliter. Equal volumes of protoplast suspension of both species were mixed and poured into a fusion chamber.

Protoplast fusion

Protoplast fusion was performed electrically using a Shimazu Somatic Hybridizer SSH-2 (Shimazu Corp, Kyoto, Japan) with a fusion chamber FTC-04 (effective volume = 1.6 ml, distance between electrodes = 4 mm). Protoplasts were subjected to an AC field strength of 1 MHz, 100 $V.cm^{-1}$, and as soon as short protoplast chains were visible, a DC pulse of 1 $kV.cm^{-1}$ was applied for 60 μs.

Protoplast culture

Fused protoplasts were cultured at a density of 5×10^4 with a liquid NTH medium supplemented with 1 mg/l NAA, 0.2 mg/l 2,4-D, 0.5 mg/l Zeatin, 250 mg/l casaminoacids, 10% coconut water, 1% sucrose and 0.5 *M* mannitol under dim light at 25 °C. NTH medium contains 0.5 mg/l pyridoxine · HCl, 0.5 mg/l nicotinic acid and 2.0 mg/l glycine in addition to inorganic salts and vitamines of Nagata and Takebe's medium (Nagata and Takebe 1971). Approximately 6 weeks later colonies larger than approximately 0.5 mm in diameter were transferred to a solid Y medium supplemented with 0.2 mg/l NAA, 0.2 mg/l kinetin, 3% sucrose, 0.25 *M* mannitol and 0.8% agar, and cultured under the same conditions. Y medium contains half-strength inorganic constituents of MS medium (Murashige and Skoog 1962) except for NH_4NO_3, 200 mg/l NH_4NO_3 and organic constituents of MS medium. After 2 weeks, calli were transferred to a medium for shoot differentiation that contained organic and inorganic constituents of MS medium, 0.2 mg/l NAA, 1 mg/l kinetin, 3% sucrose and 0.8% agar, and cultured under a 12-h light (ca. 4×10^5 $erg.s^{-1}.cm^{-2}$), 12-h dark cycle at 25 °C for approximately 7 weeks. The shoots were rooted on MS medium supplemented with 3% sucrose and 0.8% agar without any plant growth substances and cultured under the same conditions for 2 weeks. The plantlets were potted and grown under a 12-h light, (ca. 10^6 $erg.s^{-1}.cm^{-2}$, 20 °C), 12-h dark (15 °C) cycle for 2 weeks before their transfer to greenhouse conditions. After vernalization at 9 °C for 30 days under continuous light (ca. 10^6 $erg.s^{-1}.cm^{-2}$) plants were transferred to a greenhouse and cultivated under long-day conditions at 15–25 °C.

Chromosome counting

The chromosome numbers of the regenerated plants were counted using pollen mother cells or root-tip cells according to the standard method of acetorcein staining or of DAPI (4'6-diamidino-2-phenylindole dihydrochloride) staining, respectively.

Isozyme analysis

Phosphoglucose isomerase (PGI) isozyme analysis was performed using a protein extract from leaves that was electrophoresed on a non-denaturing polyacrylamide gel according to Hirai and Kajiura (1987). PGI detection was following Vallejos (1983).

DNA isolation and hybridization analysis

Total DNA was extracted from young leaves according to Appels and Moran (1984). The DNA was digested, electrophoresed on agarose gels and then transferred to a nylon membrane (Hybond N^+, Amersham) using standard methodology (Maniatis et al. 1982). Probe DNA was labeled with digoxigenin-dUTP. The labeling of the probe, hybridization and the detection of the hybridized probe was done according to the supplier's manual (DNA Labeling and Detection Kit, Nonradioactive, Molecular Biology, Boehringer Mannheim). A nuclear 17S ribosomal RNA gene from rice (rDNA, Takaiwa et al. 1984), two *Bam*HI fragments from tobacco chloroplast DNA (cpDNA) (*Ba*1 and *Ba*2, Sugiura et al. 1986) and two pea mitochondrial genes, 26S ribosomal RNA (26S, A. Morikami and K. Nakamura, personal communication) and the alpha subunit of ATPase (*atpA*, Morikami and Nakamura 1987) were used as probes.

Clubroot disease resistance test

Clubroots were collected from infected Chinese cabbages (*Brassica campestris*) cultivated in ground vigorously infected with the disease located on a farm at Yuki, Japan, during December 1989. A spore suspension of the pathogen, *Plasmodiophora brassicae*, was prepared from the clubroots using the method described by Yoshikawa (1981). Roots of young plants were dipped into the suspension of spores (5×10^7 spores/ml) for 1 min. The plants were then potted with soil mixed with the spores (5×10^6 spores/g soil) and grown in a greenhouse at 25–30 °C (day) and 22 °C (night) for 50 or 69 days. The roots were then pulled up and observed for clubroot formation.

Results

Morphology of hybrid plants

We obtained 40 plants, 39 of which were from fusion with IOA-treated cauliflower protoplasts, named "cis", and one from the untreated cauliflower protoplasts, named "cos". Of these 40 plants, 37 were hybrid-type cis plants with leaves intermediate in shape but with flowers larger than both parents (Fig. 1). The petals were usually white, but sometimes they were pale violet, thus resembling the petals of the Japanese radish. Petals of the cauliflower are always yellow. The remaining 3 plants resembled the cauliflower in appearance.

Molecular characterization of somatic hybrid plants

Phosphoglucose isomerase (PGI) was suitable for discriminating between Japanese radish and cauliflower by

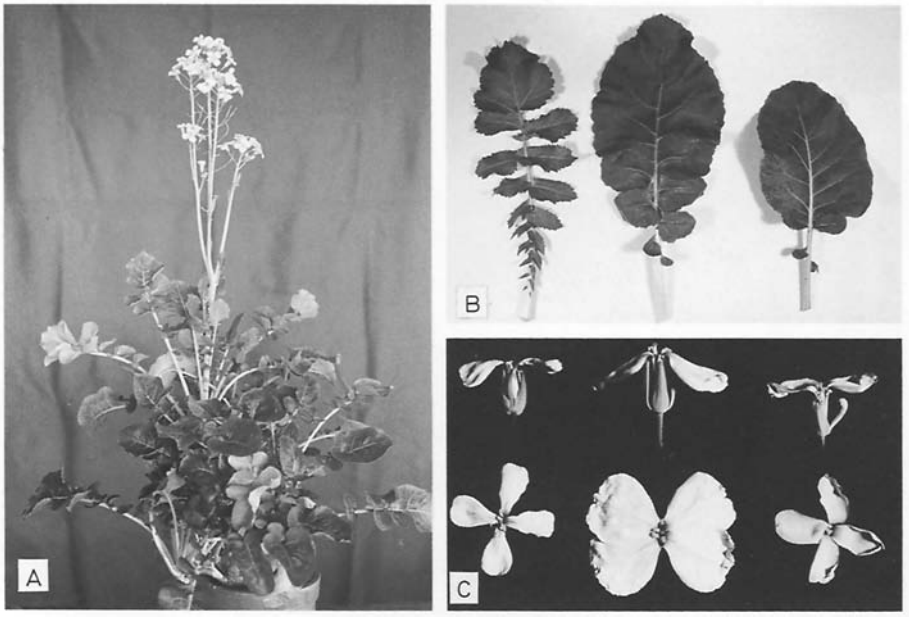

Fig. 1 A–C. Morphological features of a somatic hybrid. **A** Whole plant of somatic hybrid cis 2–2. **B, C** Leaves (**B**) and flowers (**C**) of Japanese radish (*left*), somatic hybrid (*middle*) and cauliflower (*right*)

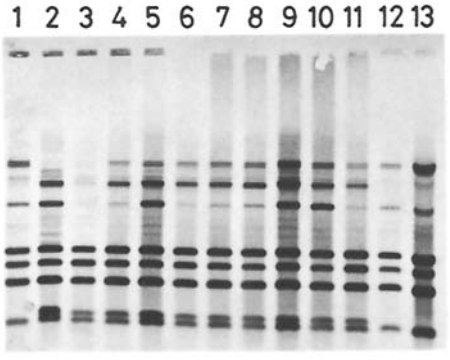

Fig. 2. RFLP analysis of total DNA, digested with *Eco*RI, of hybrids and parents. A nuclear genome-coded gene, rDNA, was used as the probe. *Lane 1* Cauliflower, *2* Japanese radish, *3* cis 0, *4* cis 1–2, *5* cis 2–1, *6* cis 2–2, *7* cis 3–1, *8* cis 3–2, *9* cis 4–1, 10 cis 4–1–2, *11* cis 4–1–3, *12* cis 5–2, *13* cos 1–3

isozyme analysis. Seven regenerated plants, all of which were of the hybrid type, were analyzed for PGI isozyme. All of them showed all of the bands of both parents (Table 1).

Nuclear, chloroplast and mitochondrial genomes were characterized using the nuclear rDNA gene (rDNA), two cpDNA genes (*Ba1* and *Ba2*) and two mitochondrial genes (26S and *atpA*), respectively, as probes. When the DNAs of both parents were digested with *Eco*RI and analyzed and compared using the rDNA probe, 2 bands specific to the Japanese radish and an additional 2 bands of the cauliflower were detected along with other bands common to both species. These 4 bands, specific to either parent, were all detected in the DNAs of all hybrid-type plants tested. However, in the DNAs of the cauliflower-type plants, only the cauliflower hybridization pattern was detected (Fig. 2, Table 1). We thus concluded that the hybrid-type plants were true hybrids, but that the cauliflower-type ones contained only the genome of the cauliflower.

Table 1. Classification of the genomes of the nucleus, chloroplasts and mitochondria of the regenerated plants by isozyme and RFLP analyses

Plant	Nucleus		Chloroplast		Mitochondria	
	PGI[a]	rDNA[c]	*Ba1*[c]	*Ba2*[c]	26S[c]	*atpA*[c]
Cis 0	H[b]	H	R[b]	R	R	R
Cis 1–2	H	H	R	R	R	H
Cis 1–3	–	H	R	R	H	H
Cis 2–1	H	H	R	R	H	H
Cis 2–2	H	H	R	R	R	H
Cis 3–1	H	H	R	R	C	H
Cis 3–2	H	H	C[b]	C	R	R
Cis 4–1	–	H	R	R	H	R
Cis 4–1–2	H	H	R	R	H	R
Cis 4–1–3	–	H	R	R	R	R
Cis 5–2	–	C	C	C	C	C
Cis 1–3	–	C	C	C	C	C

[a] PGI, isozyme of phosphoglucose isomerase
[b] Type of genome: H, hybrid-type; R, Japanese radish-type; C, cauliflower-type
[c] Probes used in RFLP: rDNA, rice ribosomal DNA; *Ba1* and *Ba2*, *Bam*HI fragments of tobacco chloroplast DNA; 26S and *atpA*, 26S ribosomal DNA (26S) and ATPase α-subunit gene (*atpA*) of pea mitochondria

Chloroplast type

When, the DNAs of both parents were digested with *Eco*RI and analyzed and compared using *Ba1* as a probe,

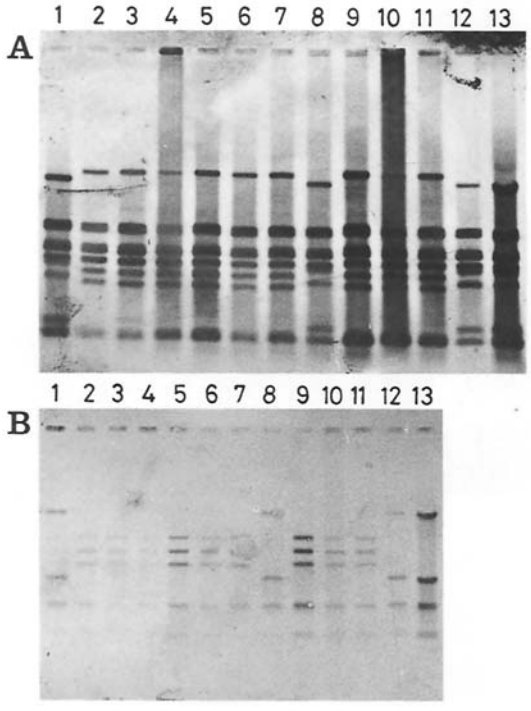

Fig. 3 A, B. RFLP analyses of total DNA, digested with *Eco*RI, of hybrids and parents. Tobacco cpDNA fragments, *Ba1* (*A*) and *Ba2* (**B**) were used as the probes. *Lanes 1–13* were the same as those in Fig. 2

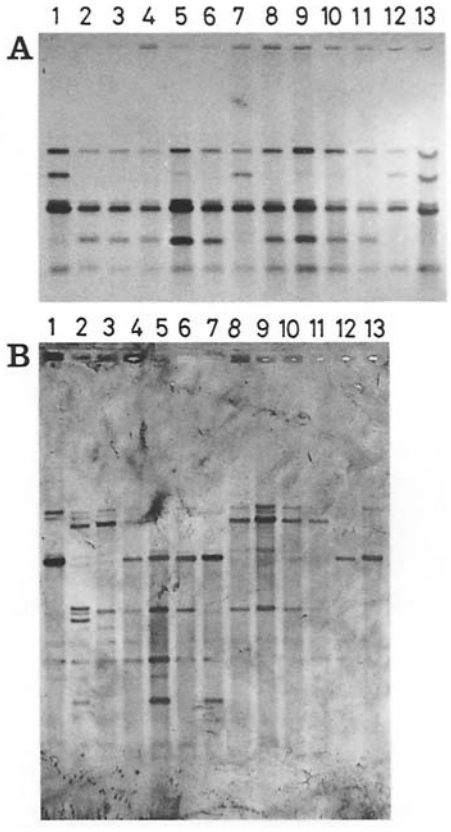

Fig. 4 A, B. RFLP analyses of total DNA, digested with *Eco*RI, of hybrids and parents. Pea mitochondrial genes, 26S (**A**) and *atpA* (**B**) were used as the probes. *Lanes 1–13* were the same as those in Fig. 2

4 cauliflower-specific bands and 3 Japanese radish-specific bands were detected with 6 common bands. When *Ba2* was used as a probe, 2 cauliflower-specific bands and 3 Japanese radish-specific bands were detected with 2 common bands (Fig. 3). All of the regenerated plants analyzed showed similar banding patterns to only one of the parents; none combined bands specific to the Japanese radish with those specific to the cauliflower. These results indicate that the regenerated plants contain chloroplasts of only one of the parents. Both probes showed consistent results with respect to chloroplast type for all plants tested. In the 10 hybrid-type plants tested, 9 proved to contain Japanese radish-type chloroplasts and 1 proved to contain cauliflower-type chloroplasts. The 2 cauliflower-type plants were seen to contain cauliflower-type chloroplasts as well (Table 1).

Mitochondria type

When 26S was used as a probe, a cauliflower-specific band and a Japanese radish-specific band were detected with other common bands (Fig. 4). Using *atpA* as a probe, a cauliflower-specific band and 5 Japanese radish-specific bands were detected in addition to other weak bands. The cauliflower-type plants showed a banding pattern similar to that of the cauliflower with both probes. In the analyses of DNA of the hybrid-type plants with a 26S probe, 5 plants showed banding patterns similar to that of the Japanese radish, one showed a banding pattern similar to the cauliflower and 4 plants combined bands of both species. With an *atpA* probe, 5 plants showed only some, not all, of the Japanese radish-specific bands, with some common weak bands and without any cauliflower-specific ones. The other 5 plants showed cauliflower-specific bands and some of the Japanese radish-specific ones in addition to common bands. Two Japanese radish-specific bands detected in Japanese radish DNA using *atpA* as a probe were not detected in any regenerated plant.

Resistance to clubroot disease

Twenty-eight regenerated plants were examined for their resistance to clubroot disease and compared to both parents (Table 2). All but 1 were hybrid-type in appearance. After an infection test, the roots of all of the hybrid-type

Table 2. Plant morphology and resistance to clubroot disease of the regenerated plants and the fusion parents

Plant	Type[a]	Club[b]	Plant	Type	Club	Plant	Type	Club
Radish	R	–	Cis 3–3	H	–	Cis 6	H	–
Cauliflower	C	+++	Cis 3–4	H	–	Cis 8–1	H	–
Cis 1–4	H	–	Cis 3–5	H	–	Cis 8–2	H	–
Cis 1–5	H	–	Cis 3–6	H	–	Cis 8–3	H	–
Cis 1–6	H	–	Cis 3–7	H	–	Cis 13	C	+
Cis 2–3	H	–	Cis 3–8	H	–	Cis 15	H	–
Cis 2–4	H	–	Cis 4–3	H	–	Cis 16–1	H	–
Cis 2–5	H	–	Cis 4–4	H	–	Cis 16–2	H	–
Cis 2–6	H	–	Cis 4–5	H	–	Cis 16–3	H	–
Cis 2–7	H	–	Cis 4–6	H	–	Cis 18	H	–

[a] Plant morphology: R, Japanese radish-type; C, cauliflower-type; H, Hybrid-type
[b] Formation of clubs on the root after infection with *P. brassicae:* –, no club formed; +, a few clubs formed; +++, more than ten clubs formed

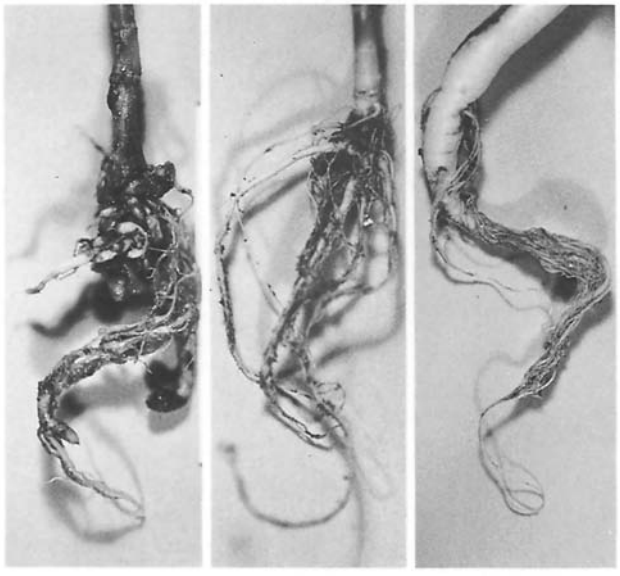

Fig. 5. Clubroot formation in the roots of a hybrid and parents after the test of resistance to clubroot disease: cauliflower (*left*), hybrid (*middle*), Japanese radish (*right*). Clubroots formed only in the cauliflower roots

plants, as well as those of the Japanese radish, were void of any clubroot infection. In contrast, on the roots of the single cauliflower-type plant and on those of the cauliflower, clubroots did form (Fig. 5). After the roots had been observed, the plants tested were replanted in pots containing sterilized soil and cultivated in a greenhouse. All of the hybrid-type plants grew normally and flowered, but the cauliflower-type plant became stunted and finally died.

Fertility

Nine plants shown to be true hybrids were examined for fertility (Table 3). They were selfed using bud pollination.

Table 3. Fertility of the regenerated plants after selfing and backcrossing

Selfing		Backcross	
Plant	Fertility	Combination[a]	Fertility
Cis 1–2	–	Cis 2–2 × C	+
Cis 1–3	–	R × cis 2–2	+
Cis 2–1	+	R × cis 4–1–3	+
Cis 2–2	+		
Cis 3–1	–		
Cis 3–2	–		
Cis 4–1	+		
Cis 4–1–2	+		
Cis 4–1–3	+		

[a] C, Cauliflower; R, Japanese radish

Seeds were obtained from hybrids belonging to clones cis 2 and cis 4, but not from those belonging to cis 1 and cis 3. Besides selfing, backcrosses to both parents were attempted. At this point, only one seed from the backcross to the cauliflower and 4 seeds from the backcross to the Japanese radish were obtained. Hybrids belonging to clones cis 2 and cis 4 had 36 chromosomes, corresponding to the total combined chromosome number of the Japanese radish ($2n=18$) and the cauliflower ($2n=18$). On the other hand, the respective chromosome numbers of clones cis 1 and cis 3 were 35 and 62. This heteroploidy is the likely explanation of the sterility of these clones. The seeds obtained from the selfing and backcrosses were sown: about 80% of the seeds from selfing and 100% of those from the backcrosses germinated and are now growing normally.

Discussion

A selection system which combines IOA treatment with culture conditions that allow only one of the parents to

regenerate was adopted. This system proved effective in the combination of the Japanese radish and cauliflower. About 95% of the regenerated plants obtained were true somatic hybrids.

Among the 10 somatic hybrids tested, 9 proved to contain radish-type chloroplasts and 1 contained cauliflower chloroplasts. The fact that all of the hybrids contained chloroplasts of either of the parents is in accordance with results reported from most of studies on somatic hybrids (reviewed by Maliga and Menczel 1986). Biased chloroplast segregation might result from differences in the chloroplast replication rates of both species. It is noteworthy that the only hybrid that contained cauliflower chloroplasts was cis 3–2, while another hybrid, cis 3–1, derived from the same clone, contained Japanese radish ones. These two hybrids originated from a single fusion product. This suggests that chloroplast segregation occurred at a late stage of culture.

The two probes for the mitochondrial genome used in the RFLP analysis showed either additive, parental-type or novel patterns, suggesting rearrangements in the mitochondrial genome. The occurrence of mitochondrial genome rearrangements or recombination in somatic hybrids has been suggested by Landgren and Glimelius (1990), Chetrit et al. (1985), Hanson et al. (1985) and Levings (1983).

Though the fertility of the hybrids was low, it is likely to be improvable through repeated selection (Ellerstrom and Zagorcheva 1977).

Our somatic hybrids between the Japanese radish and cauliflower, being fertile, crossable with cauliflower and highly resistant to the clubroot disease, are considered useful breeding material for introducing clubroot resistance into *Brassica* crops.

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