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Transfer of resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) into the *Brassica napus* L. gene pool through intergeneric somatic hybridization with *Raphanus sativus* L.

C. L. C. Lelivelt*, and F. A. Krens**

DLO – Centre for Plant Breeding and Reproduction Research (CPRO-DLO), PO Box 16, NL-6700 AA Wageningen, The Netherlands

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Summary. An intergeneric somatic hybrid was obtained through PEG-induced protoplast fusion between *Brassica napus* L. (oil-seed rape, AACC, 2n = 38) and a beet cyst nematode resistant genotype of *Raphanus sativus* L. (fodder radish, RR, 2n = 18). The hybrid nature of the regenerated plant was confirmed by flow cytometric analysis, RFLP-analysis, and chromosome counts. Southern blot analysis of total DNA using pPhcPS1 (rbc-L) as probe indicated that the somatic hybrid contains chloroplasts of *B. napus*. The mitochondrial genome of the somatic hybrid was studied more extensively using several probes and restriction enzymes. The results indicate inter- or intraspecific mitochondrial DNA recombination. Resistance to the beet cyst nematode (*Heterodera schachtii* Schm., BCN) was expressed in the hybrid at a high level.

Key words: *Brassica napus* L. – *Raphanus sativus* L. – Somatic hybridization – Nematode resistance – Restriction fragment length polymorphism (RFLP)

Introduction

Brassica napus L. is an amphidiploid species. Its subspecies *oleifera* (Metzg.) Sinsk., oil-seed rape, is one of the main oil-producing crops in Western Europe and Canada. In The Netherlands, crop rotations commonly include sugar beet as a major crop. Oil-seed rape cannot be included into such a crop rotation, because it is susceptible to *Heterodera schachtii* Schm., the beet cyst nematode (BCN). The level of resistance to BCN within *Brassica napus* L. is too low to allow selection of resistant cultivars (Harrewijn 1988). In the related species *Raphanus sativus* ssp. *oleiferus* (DC.) Metzg. (fodder radish), however, cultivars have been selected with a high level of resistance (Toxopeus and Lubberts 1979). The inheritance was studied by Baukloh (1976), who assumed a single dominant gene for resistance.

Studies of chromosome associations in MI in hybrids between oil-seed rape (AACC) and the bridging hybrid *xBrassicoraphanus* Sageret, which includes both AARR and CCRR genotypes, indicated partial homology between chromosomes of *Raphanus* (R) and *B. napus* (AC) (Dolstra 1982; Lange et al. 1989; Agnihotri et al. 1990). Sexual hybridization between *B. napus* and *R. sativus* has been reported, but only very few hybrid plants have been obtained due to the poor crossability between the two species (Chopinet 1944; Takeshita et al. 1980). Somatic cell hybridization has been found to be less impeded by pre- and post-zygotic barriers, and thus this technique may result in novel nuclear hybrids and nucleus-cytoplasm combinations.

No somatic hybrids between *B. napus* and *R. sativus* have been reported as yet. However, *B. napus* has been somatically hybridized with many other species from the *Cruciferae*, and *R. sativus*, investigated less frequently, has been used as donor of cytoplasmic male sterility in asymmetric protoplast fusions with *B. napus* (Sakai and Imamura 1990).

This paper describes the production and characterization of a somatic hybrid between *B. napus* and *R. sativus*, with the view of introducing the BCN resistance of *Raphanus* into the gene pool of *B. napus*.

* Present address: Royal Sluis, PO Box 22, NL-1600 AA Enkhuizen, The Netherlands
** To whom correspondence should be addressed

Materials and methods

Plant material

From *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk., cultivars Barrapo, Tantal, Darmor, Cascade, Jet Neuf, and accession K1 (D.J. Van der Have BV, Rilland, The Netherlands), all susceptible to *H. schachtii* Schm., and from *Raphanus sativus* (DC.) Metzg. the cv Nemex, resistant to *H. schachtii* Schm., were used.

Beet cyst nematode resistance test

Seedlings and cuttings were tested for resistance to *Heterodera schachtii* Schm. according to Toxopeus and Lubberts (1979), with slight modifications. Seeds were sown in 36-ml PVC tubes filled with silver sand, moistened with Steiner I nutrient solution, and kept in a greenhouse at a 10-h light regime, a temperature of 18 °C during the day and night, and a relative humidity of 85–90%. After 2 weeks, seedlings were inoculated with a suspension of pre-hatched L2 larvae of *Heterodera schachtii* Schm., using a veterinary syringe to inoculate with approximately 300 larvae/plant. Subsequently, temperature in the greenhouse was raised to 22 °C during the day. After 2 weeks, 2 ml Steiner I nutrient solution was added to each plant. Four weeks after inoculation, when the female nematodes had grown into cysts and had reached their maximum size, the root system was washed free of sand and examined for the occurrence of cysts.

To test the BCN resistance of eldery plants, cuttings were made that were tested using 96-ml PVC tubes and an inoculation density of 500 larvae/plant. Seedlings were used as control plants and were also tested using 500 larvae/plant. Experiments with cuttings and seedlings were replicated four times. In addition, the root size was also visually quantified on a scale of 1 (small) to 5 (large). Statistical analysis (*t*-test) was applied to the results of the BCN resistance tests.

In vitro culture

Apical meristems, axillary buds, and ovaries were sterilized in 2% (w/v) sodium hypochlorite for 10–20 min, followed by washing (three times) with sterile tap water. Seeds were rinsed in 70% (v/v) ethanol (30 s) and sterilized as described above. Except for the ovaries, all plant material was cultured on medium 1 (MS) (Murashige and Skoog 1962), 0.8% (w/v) agar, 1% (w/v) saccharose, and 1% (w/v) glucose (pH 5.8) at a 16-h light regime, 6 W/m^2 and 25 °C. Ovaries were cultured on MS medium, 5% (w/v) saccharose, 0.8% (w/v) agar, 300 mg/l casein hydrolysate, 1 mg/l indole-3-acetic acid (IAA), and 0.5 mg/l kinetin under the same culture conditions.

Mesophyll protoplasts were isolated from in vitro grown plants. In addition, protoplasts were also isolated from plants growing on medium 1, supplemented with 1.5 mg/l norflurazon (SAN 9789), to inhibit chlorophyll formation. Protoplasts were isolated and cultured according to Pelletier et al. (1983), at a density of 5×10^4 protoplasts/ml in medium Pelletier-B (2 ml per 6-ml petri dish, Greiner, TC quality). After approximately 1 week, the protoplast suspension was diluted 1:1 with medium Pelletier-C and 2 weeks after isolation the microcallus suspension was diluted 1:1 with Pelletier-D. After 3 days the number of dividing cells per 1,000 cells was counted. Plating efficiency was determined 3 weeks after isolation by counting the number of developing microcalli per plate.

Green calli, 4–5 weeks old with a diameter of 0.5 to 1 mm, were transferred to medium MS 11 [MS, 1% (w/v) saccharose, 0.8% (w/v) agar, 1 mg/l 6-benzylaminopurine (BAP), and 0.1 mg/l α-naphthaleneacetic acid (NAA)] for callus growth, and transferred after 1–2 weeks to regeneration medium Pelletier-E (26 calli/plate). Regenerants were transferred to medium 1, and after rooting plants were transferred to the greenhouse.

Protoplast fusion

For PEG-induced protoplast fusion, SAN-treated mesophyll protoplasts were stained with fluorescein diacetate (FDA) by adding 30–40 µl FDA solution (5 mg FDA/ml in acetone) to 20 ml enzyme solution at the beginning of the protoplast isolation procedure. Isolated protoplasts of both SAN-treated and non-treated plants were resuspended each in 1 ml W5 medium (Menczel and Wolfe 1984) and after protoplast yield was determined, a total of 5×10^5 protoplasts of each parent was mixed at a 1:1 ratio. The protoplast mixture was transferred to a plastic centrifuge tube (TC quality, Greiner), washed once, pelleted at $35 \times g$, and resuspended in approximately 0.2 ml of W5 medium. To this protoplast suspension, 0.4 ml of a polyethylene glycol 6,000 (PEG), mixture was added in big droplets, and the tube was incubated for 10–25 min at 18–20 °C, without shaking. Protoplasts were either incubated in a glycin/NaOH buffer (pH 10.0) containing 15% (w/v) PEG, 60 m*M* $CaCl_2 \cdot 2H_2O$, 90 m*M* mannitol, 25 m*M* glycine, and 10% (v/v) dimethylsulfoxide (DMSO) (Thomzick and Hain 1988) and subsequently rinsed thoroughly three times with washing solution (W5 medium supplemented with 50 m*M* morpholinoethane sulfonic acid, pH 5.5) (method A), or they were incubated in a PEG solution [45% (w/v) PEG in 12 m*M* $CaCl_2 \cdot 2H_2O$ solution, pH 5.8] for 10–25 min and subsequently rinsed with a glycin/NaOH buffer (pH 10.4) containing 50 m*M* $CaCl_2 \cdot 2H_2O$ (method B) (Uijtewaal 1987). Cells were plated at a density of 1×10^5 to 2×10^5 protoplasts/ml in medium Pelletier-B in 6-ml petri dishes (2 ml/petri dish). Fused protoplasts and calli were cultured as described above. Fusion frequency was determined as the number of fusion products per 500 cells.

Chromosome counts and pollen viability

Root tips from plants grown in the greenhouse were pretreated and fixed as described by Jochemsen and Mlyniec (1974), stained with Feulgen, and examined under the light microscope. Flower buds were pretreated in 96% (v/v) ethanol:ferripropionic acid (3:1, v/v) for 3 weeks and subsequently transferred to 96% (v/v) ethanol. Anthers were either stained in Snow's solution (Snow 1963) for 6–8 h at 60 °C or squashed directly in acetocarmine and examined under the light microscope. Pollen viability was estimated by staining freshly collected pollen in a solution containing 9% (w/v) sucrose and 0.5 mg/ml FDA. Viability frequency was determined under the UV microscope as the number of yellow/green fluorescent pollen in 200–300 pollen grains.

Southern blot analyses

Total DNA was extracted from leaves of plants grown in the greenhouse according to the method of Dellaporta (1983). Total plant DNA (5–10 µg) was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I, and *Xho*I according to Kreike et al. (1990). Southern blotting transfer of the DNA onto nitrocellulose (Kafatos et al. 1979) or nylon membranes (Hybond N, Amersham) and crosslinking of the DNA was carried out according to Kreike et al. (1990).

Several heterologous nuclear, chloroplast DNA (cpDNA), and mitochondrial DNA (mtDNA) sequences were used as probes in hybridizations. The maize cytochrome *C* oxidase subunit 1 (*coxI*), cytochrome *C* oxidase 2 (*coxII*), apocytochrome *B* (*cob*), and the alpha subunit of F_1 ATPase (*atpa*) were provided by the University of Edinburgh, UK (Prof. C. Leaver). The

maize ATPase subunits 6 (*atp6*) and 9 (*atp9*) were a gift from Dr. C. S. Levings III (North Carolina State University, USA), and cytochrome *C* oxidase 3 (*coxIII*) was supplied by Dr. A. Brennicke (Institute for Gene Biological Research, Berlin, FRG). The pea ribosomal DNA probe (rDNA, a 4.0-kb *Eco*RI subclone in pACycl184 from a partial genomic library of *Pisum sativum* cv Rondo) and the chloroplast probe pPhcPS1(rbc-L) were obtained from Dr P. Zabel (Agricultural University Wageningen, The Netherlands).

The probe DNA was labelled non-radioactively with digoxigenin-dUTP, hybridized to target DNA, and visualized by chemiluminescence according to Kreike et al. (1990).

Flow cytometry

Pieces of leaves (1 cm^2) were chopped with a razor blade in 1 ml of a nuclear isolation buffer (10 m*M* TRIS-HCl (pH 7), 10 m*M* spermine-tetrahydrochloride, 2.5 µg/ml 4.6-diamidino-2-phenylindole (DAPI), 10 m*M* NaCl, 200 m*M* hexyleneglycol, and 0.025% Triton-X100) and filtered through a 20-µm nylon filter. After a wash with 1 ml of the same buffer, the filtrate was analyzed on a Partec PAS-II flow cytometer with a UG 5 excitation filter, TK 420, and TK 520 dichroic mirrors and a GG 435 long-pass filter. Channel analyses were performed with the standard software of the PAS-II. *B. napus* accession K1 was used as internal standard. For the determination of DNA content in the investigated plants, the ratio was determined of G0 peak position(s) on the horizontal axis compared to the *B. napus* accession K1 G0 peak position in each experiment. For example, a peak position ratio (ppr) of 0.4 indicates that the distance of the G0 peak of the object under study to the vertical y-axis ($x=0$) is 0.4 times that of the distance of the G0 peak of *B. napus* accession K1 to $x=0$ in the same experiment.

Results

Beet cyst nematode resistance tests of parental species and sterile shoot cultures

White cysts were easy to detect on root systems of susceptible *B. napus* plants. Most seedlings of *R. sativus* cv Nemex were found to be very BCN resistant, although a few plants were found with up to five cysts when tested with 300 larvae/seedling. Seedlings of *R. sativus* cv Nemex, showing no cyst formation, were selected and used for the production of sterile shoot cultures. Shoot cultures of selected *R. sativus* cv Nemex seedlings, grown on medium 1 supplemented with SAN, were used for protoplast culture and fusion experiments. From the *B. napus* cultivars, shoot cultures, produced from seeds or, in the case of accession K1, plants and cultured on medium 1, were used for protoplast culture and fusion experiments.

Protoplast isolation and culture

Two days after the protoplast isolation, first cell divisions were visible for most of the *B. napus* cultivars. The average cell division and callus formation frequencies ranged from 8.2 and 0.09% respectively, for *B. napus* cv Darmor to 43.3 and 1.61% respectively, for *B. napus* cv Barrapo. Accession K1 was found to show the highest frequency of shoot regeneration (22%), whereas the other cultivars showed an average shoot regeneration frequency of less than 1 to 2%. Accession K1 was therefore chosen for fusion experiments.

The transfer of calli from medium Pelletier-D to shoot regeneration medium Pelletier-E often resulted in browning of the cells and arrest of further callus proliferation. Transfer of calli in a thin layer of liquid medium to the solidified medium MS 11 before transfer to medium Pelletier-E had a positive effect on callus growth, while shoot regeneration was also obtained.

Mesophyll protoplasts from *R. sativus* cv Nemex were viable during only 1 week of culture in medium B and, on average, 3.6% of the plated cells divided. Sustained cell divisions and callus formation were not observed. Addition of SAN had a negative effect on protoplast yield although cell division was not affected.

Protoplast fusion

Heterokaryons could be identified as protoplasts showing both the red autofluorescence of the *B. napus* chloroplasts as well as the yellow-green fluorescein fluorescence of *R. sativus* cytoplasm.

In order to determine favourable conditions for PEG-induced fusion, two different fusion methods were investigated. With method A a maximum heterokaryon frequency of 11% was already achieved after 15 min of incubation. With method B the heterokaryon frequency increased with increasing PEG incubation time. Maximum heterokaryon frequencies (11%) were obtained after 25 min. However, both methods showed a decrease in callus proliferation and regeneration after a PEG incubation longer than 15 min. Further fusion experiments were carried out following method A. Since the regeneration experiments had shown that *R. sativus* protoplasts did not form calli, only *B. napus* or hybrid calli and plants were expected to result from the fusion experiments. With a heterokaryon frequency of about 10% and when it is assumed that culture, proliferation, and regeneration are neither negatively nor positively affected by fusion, one out of ten plants may be expected to be a hybrid plant. In total, 1 plant out of 286 regenerants (Table 1) was found to be a hybrid plant, which would indicate that the regeneration capacity of the hybrid calli was negatively affected by fusion.

Hybrid characterization

Morphological observations. Out of 286 regenerants showed hybrid characteristics such as an intermediate leaf shape and thick and wrinkled leaves. After approximately 3 months the putative hybrid flowered. Flower buds were larger than those from the parent plants and had either the typical smooth morphology of *B. napus*, or the hairy one of *R. sativus*, or were intermediate. White

Raphanus-like flowers appeared from buds that were morphologically nearly identical to *B. napus*. Petals of the hybrid flowers were usually longer than those of the flowers of the parental genotypes. The flowers on the hybrid varied between completely white, white with yellow patches, pale yellow, and even both white and yellow petals in the same flower. Purple veins, which are characteristic for *R. sativus* flowers, were not detected. Stamen development varied from normal stamens to stamens with stunted filaments and brown or rudimentary anthers. Almost all flowers possessed the full complement of six stamens and contained nectaries. Pollen production was low and less than 5% stained with FDA. The beak length of the hybrid pods was, on average, intermediate between both parental species.

Backcrosses of SH-1 hybrid cuttings with *B. napus* cv Tantal or cv Jet Neuf were not successful. Ovary culture of 107 ovaries resulted in the formation of many aborted seeds and only one well-developed seed, which did not germinate.

The morphology of the putative hybrid indicated a highly chimeric nature. However, cuttings from this plant showed a shift towards a less chimeric phenotype, as was also indicated by flow cytometric (FCM) observations, cytological studies, and BCN tests.

Table 1. Frequency of plant regeneration from protoplast-derived calli of *B. napus*, *R. sativus*, and from calli obtained after PEG-induced fusion (method A)

Experiment	Fusion (%)	Number of calli		Number of hybrids
		Tested	Regenerants	
(a) Regeneration				
B. napus K1	–	104	12	–
R. sativus Nemex	–	0	0	–
(b) Protoplast fusion				
Expt. 1	8.4	2,519	6	1
Expt. 2	8.3	2,068	102	0
Expt. 3	10.7	1,010	56	0
Expt. 4	9.8	2,188	122	0

–=not applicable

Nuclear genome. Total plant DNA was isolated from two SH-1 hybrid cuttings: cutting 1 showed a highly chimeric nature (see also cytological studies) and cutting 2, made from cutting 1, displayed a less chimeric nature. Restriction patterns of total plant DNA, isolated from both cuttings, digested with *Bam*HI and *Hin*dIII, and probed with the pea rDNA probe, showed a discriminiation between the two parental species (Table 2). For *B. napus*, five bands (5.2, 4.3, 2.5, 2.1, and 1.2 kb) were present in the *Bam*HI restriction pattern and for *R. sativus*, four bands (4.3, 4.1, 2.5 and 1.2 kb). The somatic hybrid pattern showed the full complement of all parental bands. Digestion with *Hin*dIII resulted in two restriction fragments for *B. napus* (11 and 8 kb) and two for *R. sativus* (17 and 7 kb). Again, the somatic hybrid contained a summation of the parental bands. No novel bands appeared and none of the parental bands was missing. There was no difference between the restriction pattern of cutting 1 and 2.

Organelle genome. Using a chloroplast-specific DNA fragment as probe [pPhcPS1(rbc-L)] and *Eco*RI and *Hin*dIII as restriction enzymes, it was found that the hybrid cuttings 1 and 2 contained exclusively the pattern of the *B. napus* parent (Table 2). The *Bam*HI, *Dra*I, *Eco*RV, and *Xho*I digestions showed a restriction pattern that could not discriminate between the parental cpDNA (Table 2).

Table 2. Southern blot hybridizations of total DNA of the somatic hybrid SH-1 (*B. napus* (+) *R. sativus*) digested with seven different restriction endonucleases and hybridized with several probes

Probe	Type	Enzyme						
		*Bam*HI	*Dra*I	*Eco*RI	*Eco*RV	*Hin*dIII	*Xho*I	*Sac*I
rDNA	nuclear	hybr	–	–	–	hybr	–	–
chloro	cp	*	*	BN	*	BN	*	–
coxI	mt	*	RS	RS	RS	RS	*	–
coxII	mt	*	*	*	BN	*	*	–
coxIII	mt	*	BN	*	*	*	–	*
atpa	mt	RS	RS	RS	RS	RS	RS	–
atp6	mt	*	RS	RS	RS	RS	–	RS
atp9	mt	new	new	new	new	new	–	new
cob	mt	*	*	*	BN	*	–	*

rDNA=pea rDNA probe; chloro=chloroplast probe pPhcPS1 (rbc-L); nuclear=nuclear DNA; cp=chloroplast DNA; mt=mitochondrial DNA; BN=pattern equal to *B. napus* acc. K1, RS=pattern equal to *R. sativus* cv Nemex; – no data available; * no restriction fragment length polymorphism; new=new bands; hybr=hybrid pattern

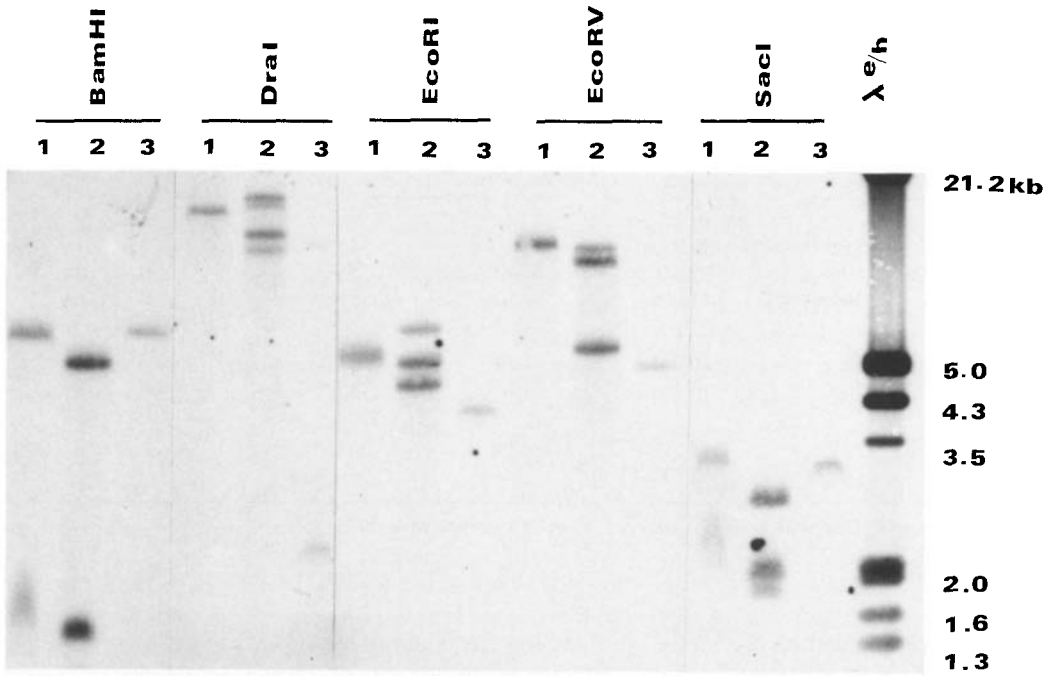

Fig. 1. Characterization of mtDNA in the SH-1 hybrid (*B. napus* (+) *R. sativus*) restricted by the enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, and *Sac*I and hybridized with the heterologous *atp9* probe. *1* = *B. napus* K1; *2* = SH-1; *3* = *R. sativus* cv Nemex. *Eco*RI/*Hin*dIII-digested lambda DNA was used as size marker

Hybridization experiments with mitochondrial probes indicated recombination between the parental species. Hybridization of total plant DNA from cutting 1, probed with *coxI*, *atpa*, and *atp6* for several restriction enzymes, showed a pattern that was identical to that of the *R. sativus* parent, whereas the use of *coxII*, *coxIII*, and *cob* resulted in the *B. napus* pattern. After hybridization with *atp9*, however, a novel banding pattern was observed which was completely different from the mtDNA pattern of either parent (Fig. 1, Table 2). The occurrence of a mixture of both parental mitochondria in the hybrid is not likely, since none of the hybridization patterns showed a summation of all parental bands. Hybridization experiments of total plant DNA, isolated from hybrid cutting 2 and probed with *coxI*, *coxIII*, and *atpa*, resulted in a restriction pattern that was identical of that of hybrid cutting 1.

Cytological studies and flow cytometry. The number of chromosomes of the first cuttings from the hybrid, used for backcrosses, morphological observations, and RFLP analyses, was found to show a chimeric pattern, since both cells with 54–56 and cells with >70 chromosomes were present in root tips. These observations were confirmed by FCM analyses of the same material. However, FCM analyses of a new series of cuttings, made from the above-mentioned chimeric cuttings, displayed a less chimeric nature. Also, chromosome counts of these plants showed a shift towards a >70 or approximately the expected number of 56 chromosomes, whereas cuttings showing tissues with both high and low numbers of chromosomes became less frequent. FCM analysis was used for detecting mixoploidy, because the number of dividing root meristem cells of some hybrid cuttings was found to be too low for the identification of chimeric tissue.

The number of chromosomes observed in 15 pollen mother cells (PMCs) varied from 58 to 62. In all cells, 26–30 bivalents, up to two trivalents and up to six univalents were observed. Meiotic irregularities were observed. At AI unequal chromosome divisions occurred, and at MII and AII restitution nuclei were observed, resulting in more than 50% dyad formation leading to unreduced pollen.

Beet cyst nematode resistance. Cuttings of the SH-1 hybrid were tested for BCN resistance (Table 3). Much variation in level of resistance was observed between cuttings. There was no relationship, however, between number of chromosomes and level of resistance. The root size of the hybrid cuttings was usually smaller than that of cuttings of *B. napus* or *R. sativus*, but bigger than that of seedlings of *B. napus*. Significantly fewer cysts were formed on the smaller root system of *B. napus* seedlings than on the larger root system of *B. napus* cuttings ($P < 0.05$). As expected, root size of the resistant species

Table 3. Cytological analyses and tests for resistance to the beet cyst nematode on the parental species *B. napus* and *R. sativus* and their somatic hybrid (SH-1). Data are means of four experiments. Letters indicate a significant difference at $P<0.05$

Plant material		Number of plants	Number of cysts/plant	Size of roots	Number of chromosomes	Flow cytometry (ppr)
Genotype	Propagation method					
B. napus						
cv Jet Neuf	seed	55	39.3 b	2.0	38	1.0
cv Jet Neuf	cutting	59	61.7 a	4.5	38	1.0
acc. K1	cutting	44	43.9 b	4.1	38	1.0
R. sativus						
cv Nemex	seed	55	0.2 d	3.6	18	0.4
cv Nemex	cutting	53	0.1 d	4.9	18	0.4
SH-1						
mean	cutting	22	5.7 c	3.4	70[a]	1.2, 2.0[a]
range			0–31	1–4	56–77	1.2–2.6

[a] =number of chromosomes and ppr values which were observed most frequently in hybrid cuttings; two ppr values indicate mixoploidy

R. sativus did not have a significant effect on the mean number of cysts formed ($P>0.95$). In all experiments, the average number of cysts produced on the root systems of hybrid cuttings was lower compared to that produced on root systems of both seedlings and cuttings of *B. napus* ($P<0.05$). Furthermore, hybrid cuttings with well-developed roots were as resistant as the *R. sativus* parent.

Discussion

PEG-induced protoplast fusion of *R. sativus* and *B. napus* resulted in one somatic hybrid.

Our results demonstrated that direct incubation of protoplasts in a PEG solution at high pH was preferable to using a later high pH treatment. This might be explained by factors such as the damaging effect of PEG after long incubation, the medium composition, or the positive effect of the interaction between PEG and Ca_{2+}/high pH on protoplast fusion.

It was estimated that with a heterokaryon frequency of about 10% (Table 1), and regeneration not affected by the fusion process, 10% of all regenerants should be hybrids. However, only 1 hybrid out of 286 regenerants was obtained. This suggests that regeneration of fusion products is reduced. In general it was found that PEG incubation had a negative effect on protoplast regeneration, since the regeneration frequency of calli from the fusion experiments was much lower than that of control *B. napus* calli (Table 1). Primard et al. (1988) investigated PEG somatic hybridization between *B. napus* and a non-regenerating *S. alba* accession, and found that in two out of three fusion experiments, the regeneration rate was low in comparison to shoot regeneration of a mixture of untreated *B. napus* and *S. alba* calli. Another explanation of the low frequency of hybrids in our experiments might be a slower growth rate of somatic hybrid calli compared to *B. napus* calli, resulting in a greater risk to die before shoots could develop. The efficiency of protoplast fusion as a method of producing hybrids might be increased by improving protoplast regeneration efficiency or by selecting hybrids at an early stage of development by means of a micromanipulator or flow cytometric sorting.

Southern blot analysis of total DNA hybridized with a pea rDNA probe showed that the somatic hybrid SH-1 contained nuclear DNA of both parents and is apparently a true hybrid. Since mitotic chromosome numbers of about 56 (the sum of the parental chromosomes) were observed, the hybrid is thought to have resulted from a one-to-one fusion (Table 3). A possible explanation for the chimeric variation in number of chromosomes in the hybrid plant could be the occurrence of disregulated cell division and callus proliferation during in vitro culture, resulting in both a duplication of chromosomes and preferential elimination of chromosomes, including those of *R. sativus*. Similar mitotic irregularities can also have occurred at the plant level, when propagating the hybrid by means of cuttings. On the hybrid, some FDA stainable pollen was formed and meiosis studies of PMCs showed the formation of a large number of bivalents. The presence of univalents at meiosis may result from somatic chromosome elimination. Trivalent formation was observed in PMCs with more than 60 chromosomes, suggesting that either homeologous chromosome association, A and/or C with R, had occurred, since partial homology between A and R and C and R has been reported (Dolstra 1982), or that preferential association of homologous duplicated chromosomes had occurred.

Marker studies and in situ hybridization studies are needed to investigate whether chromosomes from *B. na-*

pus or *R. sativus* are duplicated or eliminated in SH-1 hybrid cuttings.

All cuttings of the hybrid tested except one showed more BCN resistance than *B. napus*. Many cuttings were as resistant as the *R. sativus* parent. These results suggest that the expression of the resistance of *R. sativus* in the hybrid is strong, and is not very much influenced by the presence of the *B. napus* genome. However, the resistance studies are complicated by the genetic instability of the hybrid plant and the resulting variation in the ratio of the *B. napus* and *R. sativus* chromosomes.

Chromosomal instability has often been shown to occur in somatic hybrid plants. For example, in protoplast fusion products of *B. napus* (+) *E. sativa* (Fahleson et al. 1988) and of *S. tuberosum* (+) *S. phureja* (Puite et al. 1986) preferential elimination of chromosomes, resulting in asymmetric hybrids and also mixoploidy, was reported.

Analysis of cpDNA of the SH-1 cuttings suggested that the chloroplasts of the hybrid originate from *B. napus*, although definite proof of the origin of the chloroplast genome cannot be given, since only one probe was used for chloroplast analysis. However, chloroplast recombination in somatic hybrids is thought to be a rare event (Maliga et al. 1987).

The study of the mitochondrial genome using several probes and restriction enzyme combinations provided evidence for recombination or rearrangements in the SH-1 hybrid. Similar extensive mtDNA rearrangements, induced during tissue culture, have been reported for *Beta* (Brears et al. 1989) and *Brassica* (Shirzadegan et al. 1991). However, reports on the absence of mtDNA rearrangements in plants regenerated from protoplast-derived calli of *Brassica* are more frequent (Kemble et al. 1988; Morgan and Maliga 1987; Sakai et al. 1990). The mtDNA rearrangements reported here are more likely to have resulted from recombination between parental mtDNA in the heteroplasmic state of the hybrid cells. This is in accordance with earlier results from somatic fusion studies (Boeshore et al. 1983; Chetrit et al. 1985; Landgren and Glimelius 1990; Sakai et al. 1990).

Although a *Brassica-Raphanus* somatic hybrid with a high level of BCN resistance was obtained, the hybrid genotype showed reduced fertility and could not yet be backcrossed with *B. napus*. Therefore, further experiments are needed to optimize the production of fertile somatic hybrids between *Brassica napus* and *Raphanus sativus*, in order to be able to transfer the BCN resistance of *R. sativus* to the *B. napus* gene pool.

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References

Agnihotri A, Shivanna KR, Raina SN, Lakshmikumaran M, Prakash S, Jagannathan V (1990) Production of *Brassica napus* × *Raphanobrassica* hybrids by embryo rescue: an attempt to introduce shattering resistance into *B. napus*. Plant Breed 105:292–299

Baukloh H (1976) Untersuchung zur Wirtspflanzeneignung der Kruziferen gegenüber dem Rübennematoden, *Heterodera schachtii* (Schmidt), unter besonderer Berücksichtigung der Resistenzzüchtung. PhD thesis, Georg August University, Göttingen, pp 1–74

Boeshore ML, Lifshits I, Hanson MR, Izhar S (1983) Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol Gen Genet 190:459–467

Brears T, Curtis GJ, Lonsdale DM (1989) A specific rearrangement of mitochondrial DNA induced by tissue culture. Theor Appl Genet 77:620–624

Chetrit P, Mathieu C, Vedel F, Pelletier G, Primard C (1985) Mitochondrial DNA polymorphism induced by protoplast fusion in *Cruciferae*. Theor Appl Genet 69:361–366

Chopinet R (1944) Hybrides intergénériques *Raphano-Brassica*. Rev Hortic 116:98–100

Dellaporta SJ (1983) A plant DNA minipreparation, version II. Plant Mol Biol Rep 1:19–21

Dolstra O (1982) Synthesis and fertility of *xBrassicoraphanus* and ways of transferring *Raphanus* characters to *Brassica*. Agric Res Rep 917, Pudoc, Wageningen, The Netherlands, pp 90

Fahleson J, Råhlen L, Glimelius K (1988) Analyses of plants regenerated from protoplast fusions between *Brassica napus* and *Eruca sativa*. Theor Appl Genet 76:507–512

Harrewijn JL (1988) Screening for resistance against the beet cyst nematode, *Heterodera schachtii* SCHM., in *Brassica napus*. L. Med Fac Landbouww Rijksuniv Genet 52(2a):587–592

Jochemsen G, Mlyniec W (1974) An effective squash technique for root tips of cultivated crucifers. Genet Pol 15(4):443–447

Kafatos FC, Jones CCW, Efstratiadis A (1979) Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res 7:1541–1552

Kemble RJ, Yarrow SA, Wu SC, Barsby TL (1988) Absence of mitochondrial and chloroplast DNA recombinations in *Brassica napus* plants regenerated from protoplasts, protoplast fusions, and anther culture. Theor Appl Genet 75:875–881

Kreike CM, Koning JRA de, Krens FA (1990) Non-radioactive detection of single-copy DNA-DNA hybrids. Plant Mol Biol Rep 8(3):172–179

Landgren M, Glimelius K (1990) Analysis of chloroplast and mitochondrial segregation in three different combinations of somatic hybrids produced within *Brassicaceae*. Theor Appl Genet 80:776–784

Lange W, Toxopeus H, Lubberts JH, Dolstra O, Harrewijn JL (1989) The development of Raparadish (*xBrassicoraphanus*, 2n=38), a new crop in agriculture. Euphytica 40:1–14

Maliga P, Fejes E, Svab Z (1987) Recombination of chloroplasts in cultured plant cells. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) Plant tissue and cell culture. Alan R. Liss, New York, p 265–274

Menczel L, Wolfe K (1984) High frequency of fusion induced in freely suspended protoplast mixtures by polyethylene glycol and dimethylsulfoxide at high pH. Plant Cell Rep 3:196–198

Morgan A, Maliga P (1987) Rapid chloroplast segregation and recombination of mitochondrial DNA in *Brassica* cybrids. Mol Gen Genet 209:240–246

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rouselle R, Renard M (1983) Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191:244–250

Primard C, Vedel F, Mathieu C, Pelletier G, Chèvre AM (1988) Interspecific somatic hybridization between *Brassica napus* and *Brassica hirta* (*Sinapis alba* L.). Theor Appl Genet 75:546–552

Puite KJ, Roest S, Pijnacker LP (1986) Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *S. phureja*. Plant Cell Rep 5:262–265

Sakai T, Imamura J (1990) Intergeneric transfer of cytoplasmic male sterility between *Raphanus sativa* (cms line) and *Brassica napus* through cytoplast-protoplast fusion. Theor Appl Genet 80:421–427

Shirzadegan M, Palmer JD, Christey M, Earle ED (1991) Patterns of mitochondrial DNA instability in *Brassica campestris* cultured cells. Plant Mol Biol 16:21–37

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 38:9–13

Takeshita M, Kato M, Tokumasu S (1980) Application of ovule culture to the production of intergeneric hybrids in *Brassica* and *Raphanus*. Jpn J Genet 55:373–387

Thomzick JE, Hain R (1988) Transfer and segregation of triazine-tolerant chloroplasts in *Brassica napus* L. Theor Appl Genet 76:165–171

Toxopeus H, Lubberts JH (1979) Breeding for resistance to the sugar beet nematode (*Heterodera schachtii* Schm.) in cruciferous crops. In: Proc Eucarpia *Cruciferae* Conf, Wageningen, The Netherlands (Post Conference Edition):151

Uijtewaal, BA (1987) The production and evaluation and monohaploid potatoes (2n = x = 12) for breeding research on cell and plant level. PhD thesis, Department of Plant Breeding, Agricultural University, Wageningen, The Netherlands, pp 1–112