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Synthesis of male sterile, triazine-resistant *Brassica napus* by somatic hybridization between cytoplasmic male sterile *B. oleracea* and atrazine-resistant *B. campestris*

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Summary. Fusion of leaf protoplasts from an inbred line of *Brassica oleracea* ssp. *botrytis* (cauliflower, n = 9) carrying the Ogura (R1) male sterile cytoplasm with hypocotyl protoplasts of *B. campestris* ssp. *oleifera* (cv "Candle", n = 10) carrying an atrazine-resistant (ATR) cytoplasm resulted in the production of synthetic *B. napus* (n = 19). Thirty-four somatic hybrids were produced; they were characterized for morphology, phosphoglucose isomerase isoenzymes, ribosomal DNA hybridization patterns, chromosome numbers, and organelle composition. All somatic hybrids carried atrazine-resistant chloroplasts derived from *B. campestris*. The mitochondrial genomes in 19 hybrids were examined by restriction endonuclease and Southern blot analyses. Twelve of the 19 hybrids contained mitochondria showing novel DNA restriction patterns; of these 12 hybrids, 5 were male sterile and 7 were male fertile. The remaining hybrids contained mitochondrial DNA that was identical to that of the ATR parent and all were male fertile.

Key words: *Brassica napus* – Protoplast fusion – Triazine resistance – Cytoplasmic male sterility – Mitochondrial DNA

Introduction

Protoplast fusion provides a method to overcome natural barriers to genetic exchange between plant species. It permits the mixing and recombination of cytoplasms in species that normally exhibit uniparental inheritance of organelles and allows nuclear hybridization in sexually incompatibe individuals. From numerous experiments conducted over the last 15 years, a few generalizations describing the consequence of fusing two somatic cells have emerged (reviewed in Harms 1983; Kumar and Cocking 1987; Pelletier 1986): (1) Although it is possible to fuse any two cells, the resulting fusion product is likely to develop into normal, fertile plants only in cases where the fusion partners are closely related species. However, even in such cases, the somatic hybrid plants may be sterile because of genetic incompatibilities (O'Connell and Hanson 1986; Gleddie et al. 1986). (2) After fusion, the cytoplasms of both cells may actually mix in the heterokaryon, but this mixture is likely to be unstable. For example: (a) the plastids rapidly sort out during subsequent cell divisions at the callus stage so that plants regenerated from such calli generally contain only one of the parental types of plastid (Clark et al. 1985, 1986; Fluhr et al. 1983 a). (b) Plastids and mitochondria generally seem to sort out independently and the overall process appears to be random. Sorting out can lead, therefore, to new combinations of organelles (Barsby et al. 1987 a; Menczel et al. 1987). (c) The mitochondria of the two cells appear to interact with each other and give rise to novel mitochondrial genomes by intermolecular recombination (Rothenberg et al. 1985; Vedel et al. 1986). Mitochondria also sort out during subsequent divisions and regenerated plants may contain either of the parental types of mtDNA or novel mtDNA (Boeshore et al. 1985; Chetrit et al. 1985). (d) Intermolecular recombination can also occur between plastid genomes, but it appears to be an extremely rare event (Fluhr et al. 1983 b; Medgyesy et al. 1985).

These generalizations have emerged primarily from studies with members of the *Solanaceae*, but recent progress in the protoplast culture and plant regeneration of *B. oleracea* and *B. napus* has led to increased application

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of somatic hybridization for the agronomic improvement of these important members of the *Brassicaceae* (Pelletier et al. 1983; Schenck and Röbbelen 1982; Sundberg and Glimelius 1986; Taguchi and Kameya 1986; Terada et al. 1987).

A particularly useful application of protoplast fusion in *Brassica* is to manipulate traits such as cytoplasmic male sterility (cms), which is controlled by mitochondria, and photosynthetic herbicide resistance, which is controlled by chloroplasts (Barsby et al. 1987a, b; Menczel et al. 1987; Yarrow et al. 1986). A very stable and easily maintained form of cytoplasmic male sterility was discovered by Ogura (1968; designated "R1" by the Crucifer Genetics Cooperative) in radish and transferred to *B. oleracea*. This R1 cms cannot be used in commercial hybrid seed production of *B. oleracea* vegetables because the substitution of *Brassica* for *Raphanus* nuclei results in a temperature-sensitive chlorophyll deficiency (Bannerot et al. 1977). At temperatures below 12 °C, plants carrying the R1 cytoplasm become chlorotic, and although the chlorosis is reversed by a return to higher temperatures, the ensuing yield penalty is unacceptable. Attempts to eliminate this chlorosis by conventional breeding have been unsuccessful. As a result, other types of cms for use in *B. oleracea* have been under development for some time (Bartkowiak-Broda et al. 1979; Chiang and Crête 1985; McCollum 1981; Pearson 1972), but so far none seems to match both the stability and simple nuclear genetic maintenance of R1.

Experiments by Pelletier et al. (1983) and Menczel et al. (1987) have demonstrated that it is possible to correct the low temperature chlorosis in cms *B. napus* by introducing *Brassica* chloroplasts into the R1 cytoplasm via protoplast fusion. Similar experiments were undertaken in our laboratory to modify the R1 cytoplasm in *B. oleracea* (broccoli) by fusion with atrazine-resistant *B. campestris,* but only a single somatic hybrid was obtained (Robertson et al. 1987). To further examine cytoplasmic interactions between these two species, we have produced additional somatic hybrids using a similar combination of R1-*B. oleracea* (cauliflower) and atrazine-resistant *B. campestris*.

Materials and methods

Plant material

The *Brassica oleracea* ssp. *botrytis* used in these experiments was an inbred line (cauliflower, 7642A) carrying the R1 cms cytoplasm (Dickson 1985). The *B. campestris* ssp. *oleifera* line (cv "Candle") carried an atrazine-resistant cytoplasm (ATR) that had been backcrossed into the cultivar from wild bird's rape by Dr. W. Beversdorf, University of Guelph, Ontario, Canada.

Protoplast isolation

Cauliflower leaf protoplasts were isolated from in vitro grown plants as described (Jourdan 1988). Etiolated hypocotyl protoplasts of *B. campestris* were isolated from 4-day-old seedlings grown at 25 °C in the dark on LS medium (Linsmaier and Skoog 1965) without plant growth regulators, supplemented with 2% (w/v) sucrose, and solidified with 1% (w/v) agar. The enzyme mixture was the same as used for cauliflower, but W5 solution (Menczel et al. 1981) was used instead of SCM (0.5 *M* Sorbitol, 10 m*M* $CaCl_2$, 1 m*M* MES pH 5.8) throughout the washing procedure. The pelleted hypocotyl protoplasts were resuspended in a minimum volume of W5 solution.

Treatment with Rhodamine 6-G and iodoacetate

Prior to fusion, hypocotyl protoplasts of *B. campestris* were treated with Rhodamine 6G (Kodak, R6G) just after filtration through an 80 μm mesh nylon filter and before centrifugation. To the filtered mixture was added enough R6G (1 mg/ml stock in 10% DMSO) to give a final concentration of 10 μg/ml; the mixture was incubated at room temperature for 15–20 min. The treated protoplasts were then processed as described (Jourdan 1988). In the second of two fusion experiments (see below), leaf protoplasts were treated for 20 min with enough 10 m*M* iodoacetate (Sigma) in SCM to give a final concentration of 2.5 m*M*. After treatment, the protoplasts were processed as usual.

Protoplast fusion and culture

Two different fusion protocols were followed. In the first fusion experiment, protoplasts were fused by the plate method. Briefly, 3.8×10^5 leaf protoplasts were mixed with 7.5×10^5 hypocotyl protoplasts in a final volume of ca. 1.5 ml. The suspension was aliquoted as individual drops onto 6-cm culture plates (5 drops per plate) and the protoplasts were allowed to settle for 10 min. Five small drops of sterile 33% (w/v) polyethylene glycol 6000 (PEG) in 0.2 *M* glucose, 10 m*M* $CaCl_2$, and 0.7 m*M* KH_2PO_4 (Robertson et al. 1987) were added around the perimeter of each settled protoplast mixture and the PEG was allowed to move in. After 20 min, five drops of S5 × CM (0.5 *M* sorbitol, 50 m*M* $CaCl_2$, 1 m*M* MES pH 6.5, filter-sterilized) were added in a similar fashion and the dilution process was repeated every 5–10 min, each time doubling the number of drops of diluent until the bottom of the plate was completely covered. Five ml of S5 × CM were then added to the culture plate and gently swirled. Seven ml of the PEG/S5 × CM mixture were then removed and twice replaced with 10 ml of S5 × CM. Finally, five similar washes were done with culture medium B (Pelletier et al. 1983). After the last wash, the protoplasts were incubated in 5 ml of medium B for 6 days in the dark; the remainder of the culture procedure was as described for cauliflower leaf protoplasts (Jourdan 1988).

In the second fusion experiment, a tube fusion procedure as described by Robertson et al. (1987) was followed. After fusion, protoplasts were plated either on medium B or on medium 8p of Glimelius (1984).

Plant regeneration

Colonies derived from both fusion experiments were placed on medium E solidified with 0.22% Gelrite (Jourdan 1988), to which either no or 50 μ*M* atrazine was added. For the second fusion experiment, medium E was modified further by reducing the level of sucrose from 1% to 0.5%, and increasing the level of mannitol by an equivalent amount. Calluses selected for vigorous growth and greening in atrazine-containing medium were subcultured in the same medium to promote regeneration. Calluses that developed shoot buds were then cultured on medium F (Pelletier et al. 1983) lacking atrazine, to promote shoot development. Regenerated plantlets were hardened and transferred to soil.

Isoenzymes

Approximately 100 mg of young leaf tissue or callus were used to assay for various isoenzymes after starch gel electrophoresis. The procedures for tissue preparation, electrophoresis, and staining for enzyme activity were exactly as described by Tanksley (1979, 1980).

Chromosome counts

Flower buds of ca. 1–2 mm in length were fixed in 3:1 ferric-propionic acid for 2 days at room temperature. Immature anthers were removed, squashed in 2% aceto-carmine, and examined under 1000 × magnification.

DNA isolation, restriction, Southern transfer and hybridization

Total cellular DNA isolated from leaf blade tissue was examined by restriction endonuclease and hybridization analyses as described (Jourdan 1988).

Molecular probes

The nuclear probe, pHA2, was derived from plasmid pHA1 described in Robertson et al. (1987). pHA2 contains a HindIII-derived monomer length fragment of nuclear ribosomal DNA from *Pisum sativum*. The chloroplast probe, s8, is a cloned 21-kb fragment obtained after digestion of the *Petunia* chloroplast genome with PstI; the probe hybridizes to different size fragments in BglI digests of chloroplast DNA from *B. campestris* and *R. sativus* (Palmer et al. 1983). The mitochondrial probes were derived from the *B. campestris* mitochondrial genome (Palmer and Shields 1984); probes p5.2, p7.5, p10.2 and p12.4 were obtained from PstI digests, and probe s10.1 was obtained from SalI digests. All probes were a kind gift of Dr. J. Palmer, University of Michigan.

Determination of atrazine resistance

Resistance to atrazine in regenerated plants was determined by the tetrazolium blue assay (Robertson and Earle 1987) or by direct application of a 4.8 g/l solution of atrazine (Ciba-Geigy) to the plants.

Results

Selection of fusion products

The overall strategy in these fusions was based on a two-tier selection system: first, unfused parental protoplasts were treated with either iodoacetate or Rhodamine 6G (R6G) to prevent divisions and colony formation. Second, colonies obtained after fusion were exposed to atrazine to select for calluses which could grow in the presence of the herbicide.

Treatment with R6G selectively inhibits the mitochondria of animal cells (Gear 1974). We reasoned that if a similar effect were possible with plant cells, this dye could be used to inactivate the mitochondria of the male fertile fusion partner. To this end, we first examined the effect of R6G on the culture of hypocotyl protoplasts and found that exposure to 10 μg/ml of R6G for 15 min resulted in the collapse of protoplasts after 3–4 days, whereas untreated protoplasts regenerated walls and remained alive for up to 2–3 weeks (data not shown).

Treatment of leaf protoplasts with iodoacetate was only done in the second fusion experiment because, in the absence of this treatment, we observed that the tube-fusion procedure permitted the development of many unfused leaf protoplasts. In contrast, the plate fusion method used in the first experiment seemed to inhibit the development of unfused leaf protoplasts; in control experiments where leaf protoplasts alone were treated with PEG, we saw no development of colonies (data not shown).

Culture of protoplast-derived cauliflower calluses in the light on medium E containing 50 m*M* atrazine resulted in ca. 67% decrease in fresh weight after 47 days. This treatment also prevented greening of the calluses, particularly at the reduced levels of sucrose which otherwise lead to significant greening. Although it is expected that *B. campestris* calluses would grow on atrazine-containing medium, such evaluations could not be carried out, because hypocotyl protoplasts of *B. campestris* rarely developed into colonies on medium B and, when those rare calluses were obtained, plant regeneration occurred even more sporadically.

Given all these culture manipulations, we expected that in the first experiment, only fusion products or parental cauliflower leaf protoplasts would develop into colonies, and that the latter would then be inhibited by the herbicide on medium E. In the second experiment, we expected only fusion products and no parental protoplasts to form callus and regenerate plants.

Plant regeneration

In the first experiment, 136 calluses developed after fusion, but only 31 grew vigorously and turned green in the presence of atrazine; of these, 7 calluses regenerated shoots. In the second experiment, about 1,000 calluses were obtained after fusion, but only 24 showed sustained growth on atrazine-containing medium and, of these, 21 calluses regenerated shoots. In the end, about 100 shoots were regenerated from 28 calluses selected for vigor on atrazine-containing medium in both experiments, and one-half of these shoots grew into plants that were then characterized for nuclear traits and for composition of the cytoplasm.

Characterization of fusion products

Vegetative morphology. Among the 50 plants examined, there were two broad morphological types, distinguished primarily by the presence of trichomes in young leaves and stems, the color of the leaves, and the overall growth habit. The cauliflower plants used as source of protoplasts are glabrous; they have short internodes and somewhat light-green leaves. This light-green color is probably a result of the R1 cytoplasm they carry. The atrazine-

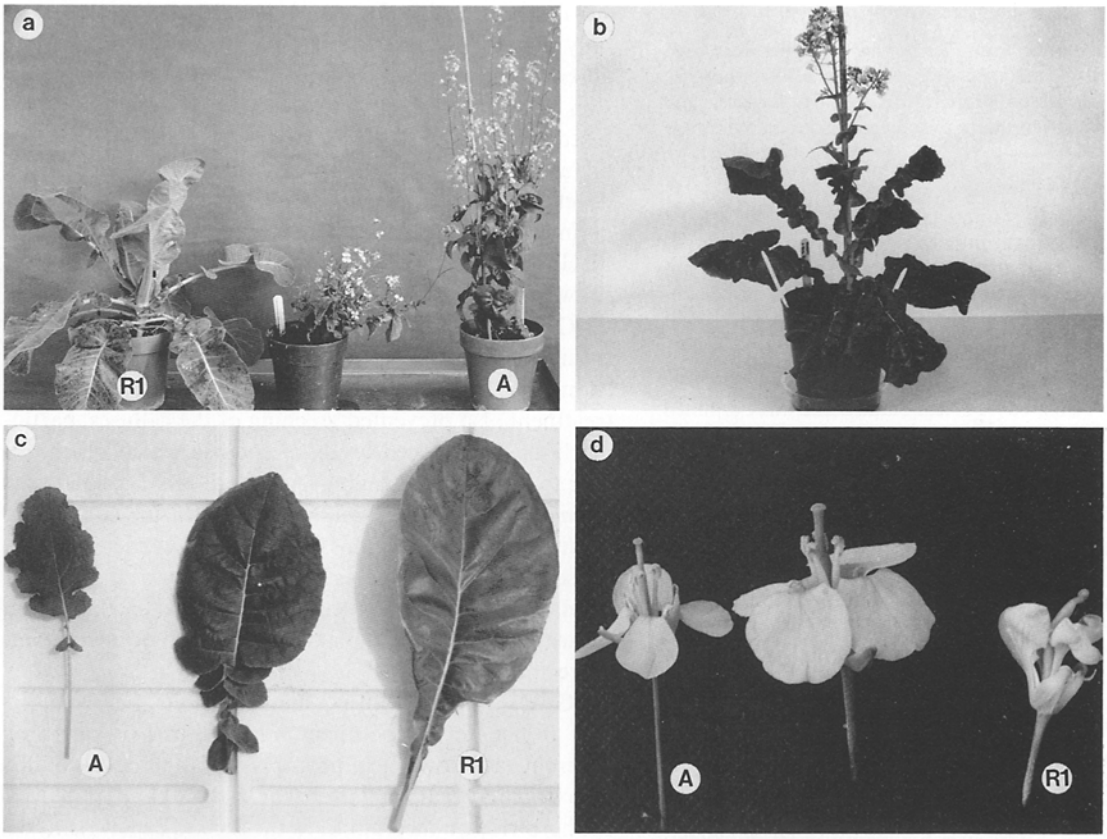

Fig. 1 a–d. Morphological characteristics of fusion products. **a** Comparison of parents and hybrid FP-6 obtained in the first fusion experiment; *R1*, *B. oleracea* cms cauliflower line 7642A regenerated from leaf protoplasts; *A*, *B. campestris* cv "Candle"-ATR regenerated from hypocotyl protoplasts. **b** Typical morphology of hybrids FP-1 to FP-5 in the first experiment. **c** Leaf morphology of the parents and of one hybrid (FP-1). **d** Flower morphology for the parents and a normal, fertile hybrid

resistant *B. campestris* plants have longer internodes, are hirsute and darker green than the cauliflower, but they are lighter green than *B. napus* plants which carry the same herbicide-resistant cytoplasm.

In the first experiment, nine plants were regenerated from seven calli. Two plants exhibited the typical morphology of cauliflower: glabrous leaves, short internodes, and light-green color. Seven plants (FP-1 to FP-7; c.f. Table 1) were morphologically similar to the *B. campestris* parent in that they were hirsute and had long internodes, but differed in that the leaves were very dark green and some plants had a petite growth habit (Fig. 1 a and b). In the second experiment, all regenerated plants were hirsute; some had dark-green leaves while others had the typical light-green leaves of the herbicide-resistant parent. Although the overall growth habit of hirsute plants from both experiments was variable, the general morphology was consistent with the characteristics of rapeseed forms of *B. napus*. Many plants had leaves with morphology intermediate between the parents (Fig. 1 c). Some plants were tall, with large, crinkled, dark-green, and relatively thin leaves, whereas others were short and had small, thick leaves. Still other plants had crinkled, thin, and light-green leaves. One plant (FP-4) showed yellow and green leaf variegation.

Floral morphology. All hirsute plants maintained in the greenhouse flowered within 3–4 months of transfer to soil. Among these plants, there were two distinct flower morphologies: one consisted of relatively large flowers with wide, overlapping petals; the other consisted of smaller flowers with narrow, non-overlapping petals. All flowers in the latter group had normal stamens with anthers full of pollen that was readily shed; plants having these flowers also had light-green leaves. Among the former group of flowers were some with normal stamens (Fig. 1 d) and others with various types of stamen abnormalities such as short, shrivelled filaments, and thin, shrivelled anthers. Abnormal pistils were often seen in flowers with abnormal stamens; these pistils had wrinkled styles or dried stigmas (not shown). In every case, each plant produced only one type of flower. The two glabrous plants obtained in the first experiment flowered much later, after producing a normal cauliflower head;

Table 1. Summary of the characteristics of fusion products. Plant: designation or number assigned to each regenerated plant. PGI: phosphoglucose isomerase isozyme forms. S – slow electromorph; F – fast electromorph; H – slow, intermediate and fast electromorphs. rDNA: hybridization pattern of the nuclear rDNA probe, pHA2, with BstEII-restricted total cellular DNA samples. ol – *B. oleracea* pattern (9-kb fragment); cam – *B. campestris* pattern (3- and 6-kb fragments); nap – equivalent to the amphidiploid *B. napus* (3-, 6-, and 9-kb fragments). Chrm No.: haploid (n) chromosome number; 19+ indicates possible aneuploid. mtDNA: restriction pattern identified with various mtDNA probes; ogu – cms pattern; cam – fertile, *B. campestris* pattern; new – novel pattern. Pollen: relative production of pollen in the flowers: (+ + +) very abundant, (+ +) moderately abundant, (+) very few and not readily shed, (−) no pollen; viability: percentage of total pollen that was stained with acetocarmine. Seed set: relative quantity of seed set after pollination with appropriate pollen. For most hybrid plants, *B. napus* "Tower"-ATR pollen was used. mod – moderate; vlow – very low

Plant	PGI	rDNA	Chrm No.	mtDNA	Pollen		Seed set
					Production	Viability	
Parents							
B. oleracea	S	ol	9	ogu	−	none	mod
B. campestris	F	cam	10	cam	+ + +	>95%	high
Fusion products							
FP-1	H	nap	19	cam	+ + +	89%	high
FP-2	H	nap	19	cam	+ + +	93%	high
FP-3	H	nap	19	cam	+ + +	95%	high
FP-4	H	nap	19	cam	+ + +	90%	high
FP-5	H	nap	19	cam	+ + +	97%	high
FP-6	H	nap	19	new	+	<20%	vlow
FP-7	H	nap	19	new	+	<15%	vlow
4402	H	nap	19	new	+ + +	86%	low
4406	H	*	19	cam	+ + +	76%	low
4415	H	nap	19	cam	+ + +	59%	low
4419	H	nap	19+	new	+	64%	none
4420	*	nap	19+	new	+	54%	none
4421	H	nap	*	new	−	none	none
4422	H	nap	19+	new	+	78%	none
4437	H	nap	19	new	−	none	vlow
4446	*	*	19	new	+ +	72%	none
4450	H	nap	19+	new	+ +	90%	vlow
4475	H	nap	19+	new	−	none	none
4506	H	nap	19+	new	+ +	24%	none

* Means that no evaluation was made for that trait in the specific plant

the flowers exhibited the typical floral abnormalities associated with the R1 cytoplasm (c.f. Fig. 1 d).

There was a wide range of pollen production and viability among the regenerated plants (Table 1). Some plants produced large quantities of viable pollen (e.g., FP-1, no. 4402), others produced smaller amounts of pollen showing low viability (FP-6, FP-7, no. 4506); still other plants produced no pollen at all (nos. 4421, 4437, 4475). A consistent finding was that plants with flowers which produced little pollen failed to shed it; the anthers had to be crushed before the individual pollen grains could be seen. Thus, among the regenerated hirsute plants there were some fully male fertile and some male sterile. Flowers of the two glabrous plants did not produce any pollen.

Isoenzyme analyses

Of various isoenzyme loci examined in the parents and fusion products, only phosphoglucose isomerase (PGI) gave consistent results that could be used for ready identification of hybrids (Fig. 2). PGI is characterized by two activity zones in the starch gels. As described by Arús and Orton (1983) for *B. oleracea, Pgi-* 1 is the more anodal band and shows no polymorphism between the species, whereas *Pgi-* 2 is polymorphic. *B. oleracea* is characterized by a slow-migrating band (S) and *B. campestris* by a fast-migrating band (F). All regenerated hirsute plants with dark-green leaves and large flowers having overlapping petals exhibited an isoenzyme banding pattern consisting of the two parental bands and the heterodimeric intermediate band (lanes 1–6 in Fig. 2). These plants are somatic hybrids (Table 1). Some of the regenerated hirsute plants obtained in the second experiment, characterized by lighter green leaves and smaller flowers with non-overlapping petals, exhibited an isoenzyme banding pattern identical to *B. campestris*. These plants appear to have regenerated from parental *B. campestris* protoplasts.

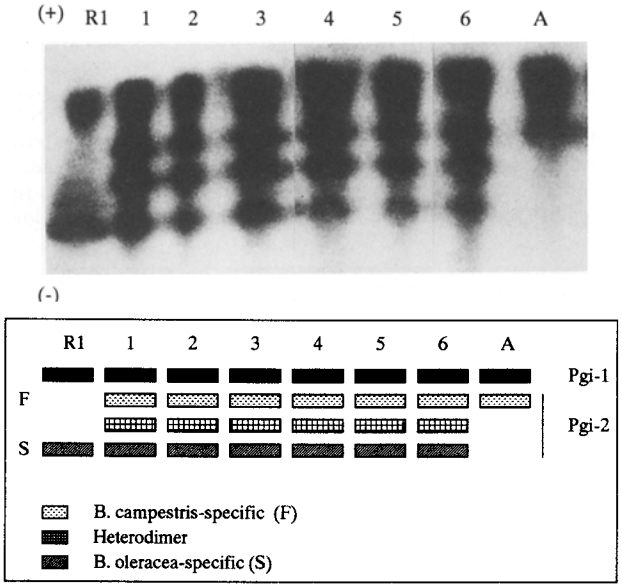

Fig. 2. Phosphoglucose isomerase isoenzymes in parents and hybrids; the lower drawing is an interpretation of the gel pictured above it. *Pgi*-1 is the anodal, plastidic form of the enzyme that is not polymorphic between the species. *Pgi*-2 is the polymorphic electromorph (Arús and Orton 1983). Extracts of young leaf tissue were used for the analysis. *R1*, cauliflower parent; *1*, FP-1; *2*, FP-2; *3*, FP-3; *4*, FP-4; *5*, FP-5; *6*, FP-6; *A*, *B. campestris* parent

rDNA analyses

The preliminary identification of regenerated plants as somatic hybrids by isoenzyme data was confirmed by analysis of rDNA sequences. Southern blot analysis of BstEII digests of DNA from *B. oleracea* yields a single band of approximately 9 kb after hybridization with probe pHA2. *B. campestris* DNA hybridizes primarily to two bands of ca. 6 and 3 kb with the same probe (Fig. 3). Somatic hybrids would be expected to contain all three bands and, indeed, many regenerated plants exhibited this pattern. In every case where the PGI data had indicated either a hybrid or a parental line, the diagnosis was supported by the rDNA analysis (e.g., lanes 12, 13, 14, and 16 in Fig. 3).

Chromosome numbers

A majority of somatic hybrids identified by isoenzyme and rDNA markers exhibited the expected addition chromosome number of the parents (*B. oleracea* n = 9, *B. campestris* n = 10, hybrids n = 19) (Table 1). In a few instances, a consistent count was not possible because the chromosome number varied between 18 and 29; e.g., hybrid nos. 4419, 4450, 4475, 4506. These plants are presumed aneuploids, but analyses of many more metaphase plates will be necessary before an accurate chromosome count is established.

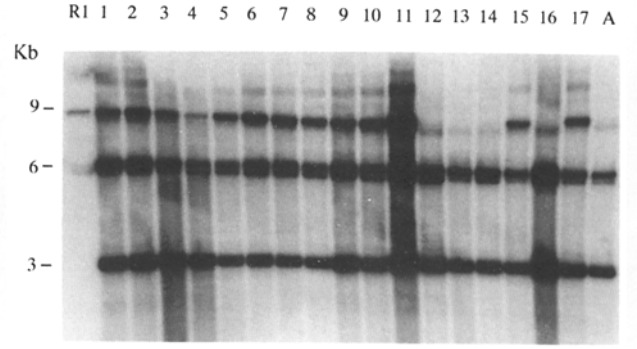

Fig. 3. Characterization of nuclear DNA in plants regenerated from the second fusion experiment. rDNA composition of the fusion products in Exp. 2 as determined by restriction of total DNA with BstEII and hybridization with probe pHA2. *R1*, cauliflower; *1*, 4402; *2*, 4406; *3*, 4407; *4*, 4415; *5*, 4419; *6*, 4420; *7*, 4421; *8*, 4422; *9*, 4437; *10*, 4446; *11*, 4450; *12*, 4405; *13*, 4467; *14*, 4469; *15*, 4475; *16*, 4477; *17*, 4506; *A*, *B. campestris*. *Lanes 12, 13, 14* and *16* represent *B. campestris* plants regenerated from unfused protoplasts

Cytoplasmic composition of regenerated plants

Chloroplasts

Low temperature-induced chlorosis. All regenerated plants transferred to soil were incubated at 10 °C for 2 weeks to determine if the susceptibility to low temperature typical of R1 chloroplasts in *Brassica* nuclear backgrounds was expressed in any of the fusion products. The somatic hybrids and the regenerated *B. campestris* plants (i.e., all hirsute regenerated plants) remained green, whereas the two regenerated cauliflower plants turned pale yellow.

Atrazine resistance. The presence of ATR chloroplasts in all hybrids was first established by evaluating herbicide resistance with the nitroblue tetrazolium test (Robertson and Earle 1987). Mesophyll protoplasts from all hybrids were able to reduce the tetrazolium dye in the presence of atrazine. This evaluation was confirmed by spraying propagules of the plants with atrazine. Only the suspected parental cauliflower plants became bleached. Thus, all hybrids were fully resistant to atrazine.

cpDNA. A probe from the *Petunia* cpDNA was used to distinguish the *Raphanus sativus* chloroplast genome (in R1 cms cytoplasm of cauliflower) from the *B. campestris* chloroplasts, after restriction of total DNA with BglI (Palmer et al. 1983). Figure 4 shows the pattern of cpDNA hybridization in the parents and in hybrids from the first experiment; identical results were obtained for the plants regenerated in the second experiment. All hirsute plants exhibited a cpDNA hybridization pattern that was identical to the ATR-*B. campestris* pattern.

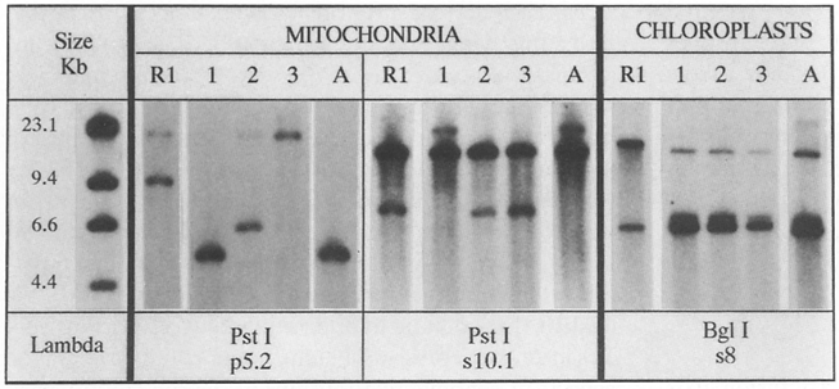

Fig. 4. Organelle composition of hybrids obtained in the first fusion experiment. Total leaf DNA was restricted with the indicated enzymes (PstI or BglI) and probed with the indicated cloned fragments. p5.2 and s10.1 are mitochondrial probes; s10.1 also hybridizes to chloroplast DNA (fragment present in all five samples). s8 is a *Petunia* chloroplast probe. *R1*, cauliflower parent; *1*, FP-1; *2*, FP-6; *3*, FP-7; *A*, *B. campestris* parent. Molecular weight makers (in kb) are indicated at left; they are derived from a HindIII digest of Lambda DNA

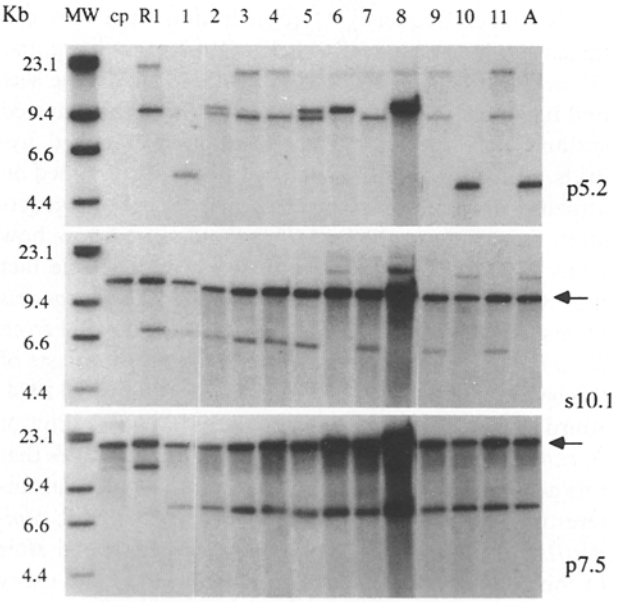

Fig. 5. Characterization of mitochondrial DNA in fusion products regenerated in the second fusion experiment. DNA was cut with PstI. On the right is indicated the mitochondrial DNA clone used as probe. The *arrows* indicate chloroplast DNA that hybridize to probes s10.1 and p7.5. *MW*, Lambda DNA cut with HindIII; *cp*, chloroplast DNA from cms cauliflower carrying the *R. sativus* cytoplasm. *R1*, cauliflower parent; *1*, 4402; *2*, 4419; *3*, 4420; *4*, 4421; *5*, 4422; *6*, 4437; *7*, 4446; *8*, 4450; *9*, 4475; *10*, 4477; *11*, 4506; *A*, *B. campestris*

Mitochondria

To determine if the occurrence of floral abnormalities and reduced pollen production in some somatic hybrids was associated with the mitochondria of the cms parent, total cellular DNA was digested with different restriction enzymes, electrophoresed, transferred to nylon membranes, and probed with cloned fragments of the *B. campestris* mitochondrial genome (Palmer and Shields 1984). Figures 4 and 5 show the mtDNA composition of hybrids from the first and second experiment, respectively. In contrast to the consistent restriction pattern seen for cpDNA, the mtDNA of somatic hybrids proved to be quite variable.

Three of the five mtDNA probes (p5.2, p7.5, s10.1) produced different patterns of hybridization between parents and hybrids after restriction with various enzymes. The other two probes (p10.2, p12.4) showed no differences in hybridization patterns either between the parents or among hybrids (data not shown).

The five male fertile hybrids produced in the first experiment (FP-1 to FP-5) appear to have a mtDNA organization identical to that of the fertile parent, because for every enzyme and probe combination examined, they revealed the same hybridization pattern as *B. campestris* (represented by lane 1 in Fig. 4). In contrast, the two hybrids with reduced pollen production (FP-6 and FP-7) exhibited novel hybridization patterns with some of the probes, and parental hybridization patterns with other probes (lanes 2 and 3 in Fig. 4). For example, in PstI digests, probe p5.2 hybridizes to new fragments of 6.9 kb in hybrid FP-6, and 16.0 kb in hybrid FP-7. Probe s10.1 hybridizes to a fragment of 7.4 kb in both hybrids; this fragment is also found in the cms parent.

The mitochondrial DNA of some hybrids obtained in the second experiment also show unique hybridization patterns (Fig. 5). Some of the mtDNA probes hybridized to species-specific bands, whereas others hybridized to unique bands. For example, DNA from hybrid no. 4422 (lane 5 in Fig. 5) hybridized to two fragments with clone p5.2, one of them corresponding to the R1-sized fragment and the other to a new fragment; to an R1 cytoplasm-specific fragment with clone s10.1; and to a *B. campestris*-specific fragment with clone p7.5. From analyses of mtDNA organization after restriction with PstI, Bg1I, and BstEII, two general hybridization patterns were discerned: (1) a pattern identical to the fertile parent (e.g., hybrids FP-1 – FP-5, 4406 and 4415; Fig. 4 and Table 1); and (2) a novel pattern that consisted of hybridization bands of either (a) new size categories (e.g., lanes 6 and 8, probe p5.2; Fig. 5) or (b) a combination of

one parental band with one probe and the other parental band with a different probe (e.g., lane 11, Fig. 5; probes p5.2 and s10.1 resemble the cms parent). None of the regenerated plants showed an overall hybridization pattern that would be equivalent to the simple physical mixture of the two parental genomes. None of the hybrids so far analyzed exhibited an overall mtDNA restriction pattern identical to the cms parent.

Discussion

We have demonstrated that by protoplast fusion it is possible to combine the nuclear genomes of *B. oleracea* and *B. campestris* with chloroplasts from the latter species and mitochondria that contain DNA sequences from both *Raphanus sativus* (R1 cytoplasm) and *B. campestris*. Some of the synthetic amphidiploid *B. napus* established by these procedures carry atrazine resistance and male sterility.

An important element in the success of these experiments was the use of a highly totipotent R1-*B. oleracea* line as one of the fusion partners; this resulted in the production of 34 somatic hybrids. Robertson et al. (1987) carried out fusions very similar to the ones reported here, but regenerated only one hybrid from 1,089 calli. This infrequent regeneration was attributed in part to low totipotency of the *B. oleracea* cms-"Green Comet" broccoli parent used. The totipotency characteristic of the R1-*B. oleracea* cauliflower used in our experiments appears to have been fully expressed in the synthetic amphidiploid even in combination with a recalcitrant genotype such as the *B. campestris* parent (Jourdan and Earle 1989). The expression of totipotency in the hybrids represents one of a series of interesting relationships that are established in these synthetic *B. napus*. For example, all hybrids resemble the *B. campestris* parent in overall growth habit, presence of trichomes, lack of central head, and short life cycle. The *B. campestris* morphology and life cycle are "dominant". When two of the somatic hybrids (FP-1 and FP-5) were evaluated for leaf protoplast culture and plant regeneration, they showed an excellent response, with high rates of division and plant regeneration (data not shown). This response contrasts with the behavior of leaf protoplasts from *B. campestris,* which have repeatedly failed to develop in our culture medium.

Thus, the *B. oleracea* totipotency trait could be described as "dominant" in the hybrids, although no formal genetic tests have yet been carried out. Terada et al. (1987) have made use of the expected regenerability of hybrids to resynthesize *B. napus* by fusion, and it is believed that totipotency of one fusion partner can, in fact, be used as a selection criterion in various fusion combinations (Harms 1983). It may be an oversimplification, however, to assume that the dominant expression of *B. oleracea*-derived totipotency would occur in all hybrids involving this species. Sundberg and Glimelius (1986), in a resynthesis of *B. napus* by fusion, manually selected the heterokaryons and obtained 450 hybrid calli, but only 4 regenerated shoots. This low frequency of regeneration, which contrasted with the ten-fold higher frequency characteristic of natural *B. napus,* was attributed to deleterious effects caused by prolonged culture of the hybrid calli, but it may also be caused by incompatible interactions between the genomes in the hybrid. It is evident that somatic hybridization between various species in *Brassica* presents a unique opportunity to examine aspects of genomic relationships at the cell culture level.

In the first experiment, 5 of 31 calli (16%) selected on atrazine-medium regenerated hybrids. This is a significantly higher frequency of regeneration than that obtained by Schenck and Röbbelen (1982), Sundberg and Glimelius (1986), or Robertson et al. (1987). In the second fusion experiment, 13 of 24 calli (54%) that showed sustained growth on atrazine-medium regenerated hybrids. However, about 750 calli were initially obtained on atrazine-medium, so a more accurate estimate of regeneration frequency might be 1.7%. We do not know how many of these 750 calli were fusion products. The fact that 8 of the 750 calli (1%) regenerated *B. campestris* plants suggests that many of the calli were derived from *B. campestris* hypocotyl protoplasts, since protoplasts of this species only rarely regenerate plants (Glimelius 1984; Jourdan and Earle 1989). Nevertheless, regeneration of *B. campestris* plants in the second experiment shows that this genotype does have some regenerative potential. Interestingly, shoots from hypocotyl protoplasts of *B. campestris* "Candle"-ATR have only been regenerated after fusion experiments. We have been unable to obtain many colonies or any shoot regeneration when the hypocotyl protoplasts are either cultured directly in medium B or 8p or after mixed culture with leaf protoplasts of cauliflower. The reason for plant regeneration under this particular set of conditions remains unclear.

Identification of hybrids among regenerated plants relied not only on a characterization of morphological parameters, but also on an assessment of nuclear and organellar traits at the molecular level. We were able to characterize species-specific nuclear sequences with a probe for part of the rDNA region (Fig. 3). This analysis showed that the hybrids contained rDNA from both parents in approximately equal amounts, whereas the regenerated parental-type plants indeed contained only the corresponding parental rDNA sequences. Although the DNA hybridizations confirmed the presence of sequences from both parents in the hybrids, this did not indicate whether these sequences were expressed. Analysis of isozymes permitted an evaluation of expressed genes from the parents; all hybrids contained, at least for the PGI locus, both parental activity zones in addition to

the heterodimer which consists of subunits derived from each parental genome.

Chromosome counts in microsporocytes of regenerated plants confirmed that the majority of hybrids contained a chromosome set from each parent. We cannot distinguish between the parental chromosomes by morphology, so at present we can only rely on their number. Schenck and Röbbelen (1982) and Taguchi and Kameya (1986) also determined that the hybrids in their experiments contained the expected 2n = 38 chromosome number. Terada et al. (1987) found a wide range of chromosome numbers in their hybrids; 60% were polyploid (6/10), possibly arising from the fusion of two *B. oleracea* with one *B. campestris*. Sundberg et al. (1987) also found wide variability in chromosome number of the hybrids; only 30% of the hybrids (7/23) had the simple addition number (2n = 38). In our experiments, 63% of the hybrids (12/19) had the diploid chromosome number. The remaining hybrids seemed to have only a few extra chromosomes and none were in the range of 2n = 58–86 as reported by Sundberg et al. (1987). The basis for these differences may lie in unique experimental conditions such as the specific genotypes involved, the procedures used for fusion, and the time between fusion and shoot regeneration. The rapid regeneration of shoots in our procedure may account for the prevalence of diploid hybrids in these experiments. However, a more exhaustive analysis of chromosome numbers in various somatic hybrids is undoubtedly necessary.

Resistance to atrazine in the hybrids appears to have been derived from the ATR chloroplasts of *B. campestris*. All hybrids contained cpDNA that had the same hybridization pattern with the 21-kb probe as the *B. campestris* parent (Fig. 4). These data suggest that herbicide resistance in the hybrids did not arise spontaneously in culture as a mutation within the R1 cpDNA or by modification of a nuclear gene (Cséplö et al. 1985). All hybrids were simultaneously resistant to the herbicide and remained green at low temperatures, suggesting that recombination between these two phenotypic markers had not occurred. However, because the analysis of cpDNA in our hybrids was rather limited, we cannot exclude the possibility that recombination may have occurred in other regions of the plastid genome. Recombination between cpDNAs in somatic hybrids has been reported in only one instance (Medgyesy et al. 1985).

Analyses of mtDNA in the hybrids showed that widespread interaction occurred between parental mitochondrial genomes in some of the fusion products. The mtDNA in 19 of the 34 hybrids regenerated in the two experiments has been partially characterized thus far. Seven hybrids had a mtDNA restriction pattern identical to the fertile parent. All seven also had an euploid set of chromosomes (2n = 38); they produced normal flowers with large quantities of pollen which was > 59% viable; they also set at least some selfed seed (Table 2). The evidence accumulated to date suggests that no interaction occurred between the two parental cytoplasms in these fusion products. The net effect has been that only the nucleus of *B. oleracea* was transferred to *B. campestris* protoplasts. It would appear as if the cytoplasms in these heterokaryons either failed to mix or, if they did, the organelle genomes did not recombine and sorted out to re-established the original combination of *Brassica* mitochondria and ATR chloroplasts.

Twelve of the 19 hybrids had novel mtDNA restriction patterns. Although it is well established that the tissue culture process itself can lead to mitochondrial genome alterations, particularly in longterm cell cultures (Chourey et al. 1986; Gengenbach et al. 1981; Grayburn and Bendich 1987; Kemble and Shepard 1984; Rode et al. 1987; Shirzagedan et al. 1989), we previously did not detect any mtDNA rearrangements in R1 cms and fertile cauliflower plants regenerated from unfused leaf protoplasts (Jourdan 1988). Morgan and Maliga (1987) observed rearranged mtDNA in calluses derived from R1-*B. napus* hypocotyl protoplasts. Such culture-induced rearrangements complicated interpretation of a novel mitochondrial genome detected in a somatic hybrid of the same line (Menczel et al. 1987). Conclusive evidence for intergenomic mtDNA recombination in R1-*B. napus* cybrids was presented by Vedel et al. (1986), and similar results were obtained by Robertson et al. (1987). In contrast, Kemble et al. (1988) have found no indication of mtDNA recombination in 334 *B. napus* cybrids and their progeny obtained from fusions between the same ATR cytoplasm used in our experiments (labelled *ctr* by them) and either the *pol* or *nap* cms cytoplasms. These workers also examined three cybrids carrying R1 mitochondria and, again, found no evidence for mtDNA rearrangement. Kemble and co-workers suggest that the degree of mtDNA rearrangement is independent of cytoplasmic type and may be related to the tissue culture system employed. However, our results clearly demonstrate that both rearranged (recombined?) and parental mtDNA can be obtained in hybrids from a single experiment, and emphasize the importance of producing many independent hybrids in order to examine the factors that may influence interaction between organelle genomes.

The association of male sterility with unique mitochondrial genomes in regenerated plants can be complicated by the various processes that are known to affect pollen production in plants derived from fusion: alterations in chromosome structure caused by the culture process, aneuploidy derived from multiple fusions or partial loss of chromosomes, and mitochondrial assortment or mtDNA recombination. Many of the regenerated hybrids had low levels of pollen production, viability, and subsequent seed set. Some of these plants also had novel mtDNA. Progeny studies with these plants are

underway to determine if the altered mtDNA and sterility are inherited maternally. Preliminary results indicate that the sterility in some of the hybrids (e.g., FP-6 and FP-7) is indeed inherited through the maternal parent (P. Jourdan, unpublished results). Such plants with altered mtDNA and male sterility may be very useful in studies of the molecular bases for cms in *Brassica*.

At present we do not have conclusive physical evidence for recombination between the parental mitochondrial genomes in the hybrids; this must await more extensive restriction site mapping. However, none of the hybrids having novel mtDNA had a pattern that could be explained by a simple addition of the parental mtDNAs. Although the probes used to analyze the mtDNA accounted for only 25% of the total mitochondrial genome, we found that the region of the DNA contained in clone p5.2 had a larger number of novel fragments than did the others. This p5.2 fragment lies adjacent to the *atp9* gene in the *B. campestris* mitochondrial genome (Makaroff and Palmer 1987) and it may be significant that this gene has been implicated in rearrangements associated with cms in *Petunia* (Young and Hanson 1987). Whether rearrangements in *atp9* are definitely associated with the R1 cms can now be explored.

The practical application of somatic hybridization for specific manipulation of mitochondrial genomes would be facilitated by the availability of an agent that could selectively inactivate the mitochondria of a fusion partner. R6G was initially thought to be such a compound. Recent reports from various laboratories (Aviv and Galun 1986; Reich et al. 1986) indicate that while R6G is indeed toxic to plant cells, it does not specifically interact with mitochondria. Our results support these conclusions because we obtained normal ATR-*B. campestris* plants from treated hypocotyl protoplasts after fusion, and because seven somatic hybrids in the two experiments had mitochondrial genomes identical to that of *B. campestris*. Thus, R6G does not appear at this time to be useful in specifically altering the mitochondrial DNA population of somatic hybrids.

The results of these experiments have clearly demonstrated the usefulness of somatic hybridization for the study of organelle genetics as well as for manipulation of desirable agronomic traits. The somatic hybrids provide material that could be useful for a more detailed study of the molecular bases for cms in *Brassica* as well as for the agronomic improvement of this important group of plants.

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