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Analysis of organelle genomes in a somatic hybrid derived from cytoplasmic male-sterile *Brassica oleracea* and atrazine-resistant *B. campestris*

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Summary. An atrazine-resistant, male-fertile *Brassica napus* plant was synthesized by fusion of protoplasts from the diploid species *B. oleracea* and *B. campestris*. Leaf protoplasts from *B. oleracea* var. *italica* carrying the Ogura male-sterile cytoplasm derived from *Raphanus sativus* were fused with etiolated hypocotyl protoplasts of atrazine-resistant *B. campestris*. The selection procedure was based on the inability of *B. campestris* protoplasts to regenerate in the media used, and the reduction of light-induced growth of *B. oleracea* tissue by atrazine. A somatic hybrid plant that differed in morphology from both *B. oleracea* and *B. campestris* was regenerated on medium containing 50 μM atrazine. Its chromosome number was 36–38, approximately that of *B. napus*. Furthermore, nuclear ribosomal DNA from this hybrid was a mixture of both parental rDNAs. Southern blot analyses of chloroplast DNA and an assay involving tetrazolium blue indicated that the hybrid contained atrazine-resistant *B. campestris* chloroplasts. The hybrid's mitochondrial genome was recombinant, containing fragments unique to each parent, as well as novel fragments carrying putative crossover points. Although the plant was female-sterile, it was successfully used to pollinate *B. napus*.

Key words: Somatic hybridization – Atrazine resistance – Mitochondrial recombinants – *Brassica* – Cytoplasmic male sterility

Introduction

Resynthesis of the amphidiploid species *Brassica napus* has been useful in plant breeding by increasing the gene pool for cultivated *B. napus* varieties and has led to the development of new fodder or vegetable crops, a few of which have been released as varieties (Prakash and Hinata 1980). *B. napus* has been resynthesized from its progenitors, *B. oleracea* and *B. campestris*, using embryo and ovule culture but the success rate is low and the results are variable, depending in part upon the particular cultivars used (Namai et al. 1980). Protoplast fusion is an alternative method of resynthesis that was first successfully used by Schenck and Robbelen (1982) and more recently by Sundberg and Glimelius (1986) and Taguchi and Kameya (1986).

An advantage of protoplast fusion is that it can result in plants with novel combinations of organelle-encoded traits. Cytoplasmically-encoded traits are usually inherited throug the maternal parent, and so it is difficult to separate chloroplast from mitochondrially-encoded traits. Fusion of somatic cells results in hybrid cytoplasms containing organelles from both parents; segregation during subsequent divisions of the hybrid cells can result in plants with new combinations of organelle-encoded traits, as well as recombinant organelle DNAs (reviewed by Fluhr 1983; Galun and Aviv 1983). In the genus *Brassica*, there are two cytoplasmically-encoded traits of agricultural interest, atrazine resistance and cytoplasmic male sterility (cms). Atrazine resistance, encoded on chloroplast DNA, has been transferred into the cultivated oil seed crops *B. napus* and *B. campestris* from an atrazine-resistant biotype of *B. campestris* (Beversdorf et al. 1980). The particular cms used in this study, the ogu cms (Ogura 1968), is associated with mitochondria (Pelletier et al. 1983) and has been crossed into *B. campestris*, *B. oleracea*, and *B. napus*.

Schenck and Robbelen (1982) relied on the poor regeneration ability of both *B. campestris* and *B.*

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oleracea compared to *B. napus* to select for somatic hybrids. We wondered whether the use of atrazine in vitro could also be used to reduce the number of atrazine-sensitive parental types. This report describes the synthesis of a male-fertile *B. napus* with atrazine-resistant chloroplasts, produced by fusion of cms *B. oleracea* (broccoli) and atrazine-resistant *B. campestris*.

Materials and methods

Plant material

Leaf and cotyledon protoplasts were isolated from *Brassica oleracea* var. *italica* cv Green Comet (GC) broccoli (Harris Moran Seed Co., Rochester, NY, USA). Most experiments used an Ogura cms line (GCcms) obtained by backcrossing an ogu cms broccoli with an inbred maintainer line derived from hybrid Green Comet. This line was developed by Dr. M. H. Dickson, New York Agricultural Experiment Station, Geneva, NY, USA. Etiolated hypocotyl protoplasts were isolated from *B. campestris* ssp. *oleifera* cv Candle, which contained atrazine-resistant chloroplasts. This line was developed by Dr. W. Beversdorf, University of Guelph, Guelph, Ontario, Canada. The rapid cycling strains of *B. napus* (CrGC12) and *B. oleracea* (CrGC3) were obtained from the Crucifer Genetics Cooperative, Madison, WI, USA.

Protoplast isolation and fusion

Leaf and cotyledon protoplast donor plants were grown from seed in 500 ml beakers with autoclaved vermiculite moistened with ½ strength MS salts (Murashige and Skoog 1962), at 25 °C with a 16 h photoperiod of 80 $\mu E/m^2/s$. Protoplasts were isolated from 3 young, fully expanded leaves of 24-day-old plants. Leaf tissue was scored with parallel cuts 1 mm apart and placed in enzyme diluted 1:8 with SCMP (0.5 M sorbitol, 10 mM $CaCl_2$, 5 mM Mes, 1 mM KH_2PO_4, pH 5.8). The enzyme stock solution consisted of 2% w/v Cellulase CELF, 0.1% Pectolyase Y-23, 0.5% Driselase, 0.2 M mannitol, 80 mM $CaCl_2$, 1 mM KH_2PO_4 and 1 mM Mes, pH 5.6. Etiolated hypocotyl protoplasts were isolated from 4-day-old *B. campestris* seedlings grown in the dark at 25 °C on medium A (Pelletier et al. 1983). About 30 hypocotyls were slit longitudinally and placed in enzyme diluted 1:4 with SCMP. After 16 h incubation at 50 rpm at 30 °C, leaf protoplasts were diluted with SCMP and hypocotyl protplasts with 0.18 M $CaCl_2$ and 1 mM Mes, pH 5.8. Protoplasts were centrifuged 5 min at 55 g (leaf) or 75 g (hypocotyl). Protoplast pellets were resuspended in 3 ml 0.5 M sucrose with 1 mM Mes (pH 5.8), a small amount of SCMP was layered on top and the tubes were centrifuged 5 min at 55 g. The floating bands from both tubes were combined and diluted with SCMP to a final concentration of 2.5 to 5×10^5/ml. The ratio of GCcms to *B. campestris* protoplasts was 1:1.3.

The procedure for fusion was derived from that of Douglas et al. (1981). Polyethylene glycol (PEG) 6,000 was prepared as a 33% w/v solution in 0.2 M glucose, 10 mM $CaCl_2$ and 0.7 mM KH_2PO_4 and filter sterilized. One milliliter of the protoplast mixture was placed in each of four 15 ml conical test tubes and 1 ml of the PEG solution was gradually added down the side of each tube and gently mixed. After 15 min, 0.5 ml SCMP was added and mixed. At intervals of 5 min, 1 ml, 2 ml, 4 ml, and 4 ml of 0.18 M $CaCl_2$ were added and gently mixed. Tubes were centrifuged 5 min at 75 g and the pellets resuspended in 4 ml SCMP. After centrifugation protoplasts were washed once in medium B (Pelletier et al. 1983). They were plated in 0.5 ml aliquots of medium B in 24-well multiwell plates (Falcon) at a concentration of 5×10^4/ml.

Plant regeneration

The procedures described by Robertson and Earle (1986) were followed, using the series of media (B-G) developed by Pelletier et al. (1983). Protoplasts were kept in the dark for 2 days and then exposed to 80 $\mu E/m^2/s$ light (provided by a combination of Gro-lux and cool white fluorescent lights) in a 16 h photoperiod at 25 °C. After 4 weeks of culture, protoplast-derived calli (p-calli) were transferred to medium E solidified with 0.22% Gelrite with or without different concentrations of atrazine. A 50 mM stock solution of atrazine (gift of Ciba Geigy) was prepared in 95% ethanol, stored at −20 °C, and added to autoclaved medium E. Shoots initiated on medium E were transferred to Gelrite-solidified media F and G without atrazine. Plants that did not root on medium G were rooted in medium containing 1% sucrose, ½ strength MS salts, 0.1 mg/l NAA and 1% agar in Magenta GA-7 boxes. All plants were hardened as described by Robertson and Earle (1986). Chromosome numbers of regenerated plants were determined from dividing microsporocytes stained with acetocarmine.

Determination of atrazine resistance

The tetrazolium blue assay (Robertson and Earle 1987) was used to determine atrazine resistance. Protoplasts were suspended in medium containing 0.6 M sorbitol, 50 mM Hepes (pH 7.6) and 10 mM $NaHCO_3$ with or without 50 μM atrazine. Nitro-blue tetrazolium (Sigma) was added to a concentration of 0.01% and the protoplasts were exposed to 150 $\mu E/m^2/s$ light for 30 min. The degree of staining of 500 protoplasts was evaluated using an inverted microscope.

Molecular characterization

Total cellular DNA was prepared from the atrazine-resistant somatic hybrid and its fusion parents by the procedure of Saghai-Maroof et al. (1984). The DNAs were further purified by two bandings in CsCl/ethidium bromide and dialysis. Restriction endonuclease digestions, agarose gel electrophoresis, bidirectional transfers of DNA fragments from agarose gels to Zetabind (AMF Cuono) hybridization membranes, labeling of recombinant plasmids by nick-translation, and filter hybridizations were performed as described (Palmer 1982, 1985).

Results

Protoplast fusion and regeneration of hybrid

The fusion procedure consistently produced 1%–6% heterokaryotic cells with transvacuolar strands from the *B. campestris* hypocotyl protoplasts and chloroplasts from the *B. oleracea* leaf protoplasts. In the experiment reported here, 1,089 p-calli showed growth on solidified medium E 4 weeks after the fusion procedure. After 5 weeks, equal numbers of these calli, as well as about 200 calli from control GCcms protoplasts not exposed to PEG, were transferred to medium E with or without 50 μM atrazine. Although unfused GCcms control protoplasts from this experiment developed to the callus stage, no shoots were regenerated on medium E with or

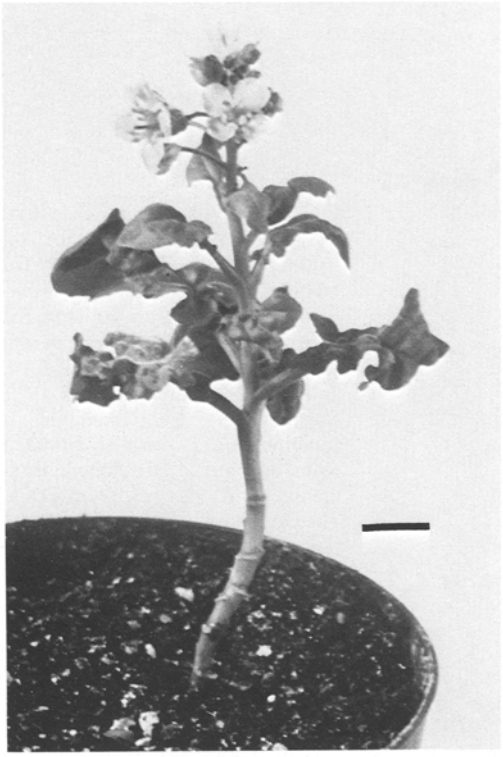

Fig. 1. Morphology of the somatic hybrid at the flowering stage. Bar = 1 cm

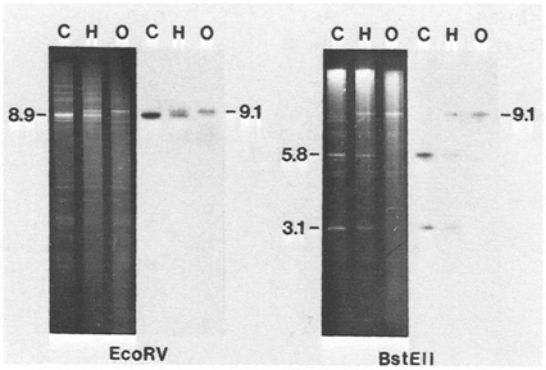

Fig. 2. Analysis of nuclear rDNA sequences in the atrazine-resistant somatic hybrid and its parents. Total cellular DNAs from the *B. campestris* parent (*C*), the hybrid (*H*), and the *B. oleracea* parent (*O*) were digested with EcoRV and BstEII and electrophoresed on a 1.0% agarose gel (left half of each panel). The DNA fragments were transferred to a zetabind filter and hybridized with nick-translated pHA1 (right half of each panel). Fragment sizes are given in kb

Table 1. Reduction of nitro-blue tetrazolium in protoplasts from atrazine-susceptible and -resistant lines of *B. napus* cv Tower and from the somatic hybrid

Protoplast source	Atrazine (μ*M*) 0	50
B. napus, atrazine-susceptible	80%[a]	0%
B. napus, atrazine-resistant	92%	86%
somatic hybrid	86%	84%

[a] Percentage of stained protoplasts in 500 protoplasts counted

without atrazine. In previous studies (Robertson and Earle 1986), GCcms also had a low morphogenetic potential. The frequency of shoot formation of the 1,089 calli from the fusion treatment was low but 4 shoots were recovered. Three were regenerated on medium lacking atrazine. They were vitreous, lacked trichomes, and were not successfully hardened.

The fourth shoot was regenerated on medium containing 50 μM atrazine. Trichomes were visible on this shoot when it was 2 cm high. As *B. campestris*, which has trichomes, does not regenerate from protoplasts on the media used and as broccoli lacks trichomes, the plantlet was tentatively identified as a somatic hybrid. The shoot did not root in medium G (without atrazine) but root formation did occur on medium containing ½ MS salts, 1% sucrose, 1% agar and 0.1 mg/l NAA. Over 30 clones derived from axillary buds of this hybrid were propagated by placing excised nodes on rooting medium. Even successfully hardened clones were difficult to grow because root development was poor. The first clone flowered one year after the fusion experiment, shortly after transfer from a 16 h photoperiod at 20 °C to continuous light of comparable intensity at 25 °C.

The morphology of the somatic hybrid (Fig. 1) differed from both parents. The leaves were rugose, and the stem was thicker than *B. campestris*. Young leaves and stems were densely covered with trichomes. As the plant matured, the density of trichomes decreased, and at flowering it completely lacked trichomes and had a waxy bloom. The plant was 10 cm tall when it flowered. Its chromosome number was 36–38, which is close to the diploid chromosome number for *B. napus* (2n = 38).

Analysis of nuclear ribosomal DNA sequences confirmed that the putative somatic hybrid does indeed possess a hybrid nucleus. Plasmid pHA1, which contains a monomer length fragment of nuclear ribosomal DNA from pea (Jorgensen et al. 1982), was hybridized to Southern blots containing total DNA from the hybrid and its parents, each digested with four enzymes (Xho I, BstE II, EcoR V, Xba I) known (J. D. Palmer, unpublished data) to distinguish the parental nuclear rDNAs. As expected, the hybrid contained the full complement of both parental sets of bands with each of the four enzymes, results for two of which are shown in Fig. 2.

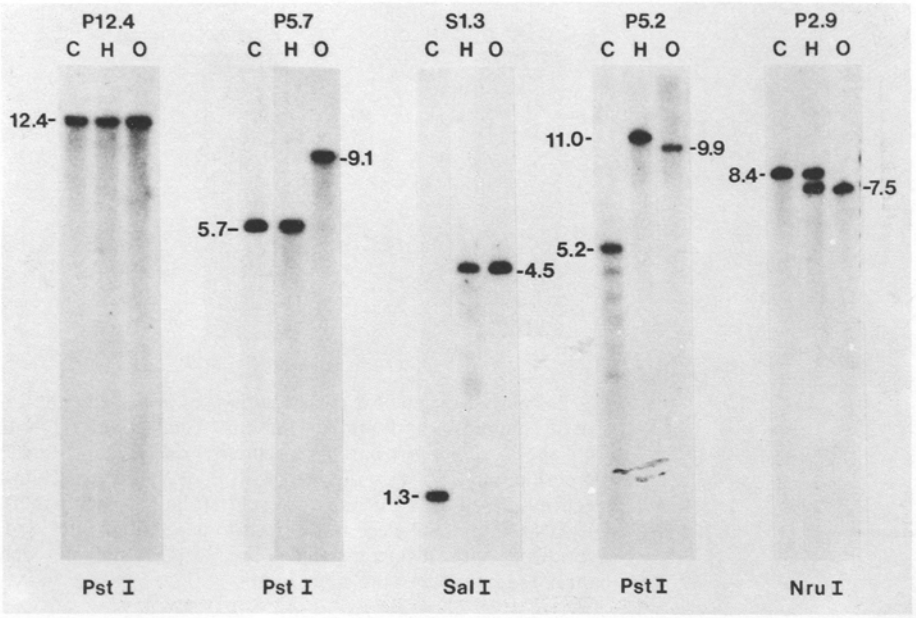

Fig. 3. Analysis of mitochondrial DNA sequences in the atrazine-resistant hybrid and its parents. Total cellular DNAs from the *B. campestris* parent (*C*), the somatic hybrid (*H*), and *B. oleracea* parent (*O*) were digested with Pst I, Sal I, and Nru I, separated on a 0.7% agarose gel, transferred to a zetabind filter, and hybridized with the indicated cloned fragments of *B. campestris* mtDNA. The clone designations indicate the type of fragment cloned ("P" for Pst I and "S" for Sal I) and its size in kb

The somatic hybrid was male-fertile, with a flower morphology similar to *B. campestris.* Although pollen from this plant was successfully used in crosses with rapid cycling *B. napus* (CrGC12), the plant was female-sterile, even when buds or open flowers were self-pollinated or pollinated from CrGC12 or CrGC3 (rapid cycling *B. oleracea*).

Analysis of organellar traits

The tetrazolium blue assay was used to determine whether the regenerated plants contained atrazine-resistant chloroplasts. In this assay, reduction of nitro-blue tetrazolium by photosystem II electron transport leads to visible staining of chloroplasts within protoplasts. Protoplasts from atrazine-resistant and atrazine-sensitive *B. napus* show comparable staining in the absence of atrazine; addition of atrazine inhibits staining of the sensitive but not the resistant protoplasts (Table 1). There was no reduction in staining of protoplasts from the somatic hybrid in medium containing 50 μM atrazine (Table 1), indicating that this plant was atrazine-resistant.

Based on its resistance to atrazine, a cpDNA-encoded trait carried by the *B. campestris* parent, we expected the hybrid to contain the *B. campestris* chloroplast genome. To confirm this expectation, total DNA from the hybrid and its parents was digested with each of four enzymes (Bgl I, Sac II, Sal I, Sma I) known (Palmer et al. 1983) to distinguish the parental cpDNAs and hybridized with appropriate cloned cpDNA fragments. With every enzyme tested the hybrid was found to contain a *B. campestris*-specific cpDNA restriction fragment or fragments (hybridizations not shown). The five restriction site mutations that distinguish the parental genomes with the four enzymes used are scattered widely in the large single copy region and inverted repeat of the *Brassica* chloroplast genome (Palmer et al. 1983). Therefore, it seems likely that the hybrid contains exclusively *B. campestris* cpDNA, although we connot rule out the possibility that the hybrid chloroplast genome contains relatively small portions of the Ogura chloroplast genome (which would have to be indistinguishable from the homologous portions of the *B. campestris* genome with the enzymes used) as the result of recombination between parental cpDNAs.

In contrast to the straightforward compostion of the hybrid's nuclear and cloroplast genomes, its mitochondrial genome was found to consist of a complex mixture of the parental mtDNAs. Figure 3 shows the hybridization patterns of five representative *B. campestris* mtDNA clones (Palmer and Shields 1984) to total DNAs from the hybrid and its parents. P12.4, a clone containing a 12.4 kb Pst I fragment, hybridized to identically-sized fragments in the two parents and in the hybrid. In contrast, each of the other four clones hybridized to a single species-specific band in each of the parental DNAs. Moreover, each of these clones revealed a different pattern of fragments for the hybrid relative to its parents. P5.7 hybridized to a single fragment in the hybrid of the same size as in the *B. campestris* parent, while, conversely, S1.3 hybridized to a single *B. oleracea*-specific fragment in the hybrid (Fig. 3). Thus, it appears that the mitochondrial ge-

nome of the hybrid is recombinant, containing specific portions of each of the parental genomes.

More complex results, also consistent with the recombination hypotheses, were observed with the other two clones used in Fig. 3. P5.2 hybridized to a single fragment in the hybrid; this fragment differs in size from either of the parental-specific fragments. We interpret this hybrid fragment as containing a crossover point for recombination between the parental genomes, i.e., this fragment should contain a portion of each parental genome. P2.9 hybridized to two fragments in the hybrid, one corresponding in size to the *B. campestris*-specific fragment and the other to the *B. oleracea*-specific fragment (Fig. 3). Thus, the hybrid appears to contain both parental sequences from this region of the genome, so that sequences present only once in either parent are duplicated in the hybrid. This result is consistent with simple models of recombination between parental genomes if we assume that the 8.4 kb and 7.5 kb parental homologous sequences are located on opposite sides of the recombining chromosomes relative to a point of reciprocal crossover. Such a topology is easily imagined, given that the mitochondrial chromosomes from the parents are known to be highly rearranged as the result of a dozen or so large inversions (J. D. Palmer and L. A. Herbon, unpublished results).

Discussion

One goal of this work was to develop improved methods for obtaining *Brassica* somatic hybrids. Although the frequency of shoot formation was low (less than 1% of the calli produced shoots) one of them was a somatic hybrid. Another goal, to begin study of the genetics of cms, was accomplished in that the male-fertile somatic hybrid contained a mt genome recombinant between male fertility- and male sterility-encoding mt.

Our previous experiments (Robertson and Earle 1986) indicated that GCcms P-calli formed shoots less frequently than p-calli from fertile, hybrid GC. In the experiment reported here, about 200 calli from unfused control GCcms protoplasts formed no shoots on medium E and protoplasts from unfused *B. campestris* divided but did not develop to a point where they could be transferred to agar. Other investigators have also noted that *B. campestris* does not regenerate easily from protoplasts (Xu et al. 1982; Lu et al. 1982; Glimelius 1984). Only 0.7% of calli from protoplasts treated with PEG (3/545) formed shoots on medium E without atrazine. The low totipotency of both parental species may in part explain the low frequency of regeneration in the fusion experiment and may also have contributed to the selection of the *B. napus*-like fusion product.

Only one plant regenerated on medium containing atrazine; this plant was atrazine-resistant, male fertile, and had a diploid chromosome number similar to that of *B. napus*. Pelletier et al. (1983) used *B. napus*, which shows high frequency regeneration from protoplasts, as a source of atrazine resistance. Protoplasts from atrazine resistant *B. napus* and cms *B. napus* were fused but only 2 out of 85 regenerated plants contained atrazine resistant chloroplasts.

Experiments using 4-week-old p-calli from fertile, hybrid Green Comet showed that 1–50 μM atrazine in medium E with 1% sucrose reduced the growth rate in the light by a factor of 4, to a rate comparable to dark-grown calli (data not shown). Grant et al. (1983) also reported that atrazine reduced the growth of atrazine sensitive *B. napus* callus to levels of dark-grown callus and that growth of atrazine-resistant *B. napus* callus was not inhibited by atrazine. As the number of shoots regenerated in the fusion experiment reported here was small (four), it was not possible to determine the effectiveness of atrazine as an in vitro selective agent.

Schenck and Robbelen (1982) fused protoplasts from 1,236 different combinations of varieties and cultivars of *B. oleracea* and *B. campestris* but were able to regenerate only 3 true synthetic *B. napus* plants. The experiments reported here used only two lines, derived from the cultivars Green Comet and Candle, and resulted in the regeneration of one synthetic *B. napus*. Taguchi and Kameya (1986) also regenerated two synthetic *B. napus* derived from fusion of leaf protoplasts from two lines: Chinese cabbage (*B. campestris*) and cabbage (*B. oleracea*). Although they found that calli derived from fusion products showed more vigorous development than calli derived from unfused protoplasts, we were unable to detect a difference between development of calli from unfused GCcms protoplasts and the protoplast fusion products. We used different media for protoplast development, and so our results are not strictly comparable. Sundberg and Glimelius (1986) have also regenerated *B. napus* from fusion of protoplasts from *B. oleracea* and *B. campestris*. Using micromanipulation they were able to select fusion products at an early stage and obtained 450 hybrid calli and about 1% shoot formation. This low frequency of shoot formation emphasizes the need for good selection techniques.

The rDNA analysis indicated that at the time of analysis genome components from both parents were present in approximately equal amounts in the somatic hybrid. This technique, which has also been used in tobacco (Uchimaya et al. 1983), was especially useful in determining the nuclear constitution of the somatic hybrid since poor root development made root tip

chromosome counts difficult and the plant took 1 year to flower. The estimated chromosome numer of the somatic hybrid was 36–38. The rDNA analysis eliminated the possibility that the somatic hybrid was a tetraploid *B. campestris* (4n = 40) rather than a diploid *B. napus* (2n = 38).

The hybrid regenerated in this study was self- and female-sterile, although it was successfully used to pollinate rapid-cycling *B. napus*. The three synthetic amphidiploids regenerated by Schenck and Robbelen (1982) were self-fertile although the number of seed produced was small.

The tetrazolium blue assay provided a convenient method for determining atrazine resistance. Since it requires only a small amount of tissue, it could be performed as soon as the leaves from the hybrid showed normal (non-vitreous) morphology. Results from Southern hybridizations confirmed those of the tetrazolium blue assay by showing that the atrazine-resistant somatic hybrid contained *B. campestris* chloroplast DNA. Although Medgyesy et al. (1985) reported recombination of chloroplast DNA in an interspecific *Nicotiana* somatic hybrid, in all other somatic hybrids chloroplast DNA recombination has not been detected (Fluhr et al. 1983).

The hybrid's mitochondrial genome contained restriction fragments specific to each parent, as well as novel fragments unlike those of either parent. Although the latter observation alone could be explained by internal rearrangement of mtDNA, as sometimes occurs during tissue culture (Gengenbach et al. 1981; Kemble et al. 1982; Kemble and Shepard 1984), the two observations together strongly suggest recombination between parental mtDNA, leading to the creation of a "hybrid" mitochondrial genome. Evidence for mtDNA recombination in somatic hybrids has also been described in tobacco (Belliard et al. 1979; Nagy et al. 1983; Galun et al. 1982), *Petunia* (Boeshore et al. 1983), *Brassica* (Chetrit et al. 1985) and *Daucus* (Matthews and Widholm 1985). The strongest evidence in favor of the recombination hypothesis is that of Rothenberg et al. (1985), who cloned a novel mtDNA fragment from a *Petunia* somatic hybrid and showed that it contained restriction sites from both parents.

Our current studies are taking advantage of the relatively small and well-understood mitochondrial genome of *Brassica* species (Palmer and Shields 1984) in an attempt to determine the complete structure of the recombinant mt genome in this hybrid and to identify the exact points of crossover. Furthermore, by analyzing the mitochondrial genomes of additional hybrids, both male-fertile and sterile, we hope to identify specific portion(s) of the genome that may be involved in sterility, as Boeshore et al. (1985) were able to do in *Petunia*.

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