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The combination of Polima cms and cytoplasmic triazine resistance in *Brassica napus*

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Summary. Protoplast fusion was used to combine cytoplasmic triazine resistance (ctr) and Polima type cytoplasmic male sterility (cms) in *Brassica napus*. The cybrids produced constitute the major biological input required for the production of commercial single-cross hybrid rapeseed bearing cytoplasmic triazine resistance. The results also indicate that Polima cms is associated with the mitochondrial genome.

Key words: *B. napus* – Protoplast fusion – Cybrids – Triazine resistance – Male sterility

Introduction

Evaluations of hand-crossed rapeseed (*Brassica napus*) hybrids have indicated that hybrid seed may provide as much as a 40% increase in yield, relative to conventional varieties (Schuster and Michael 1976; Sernyk and Stefansson 1983). In addition, where weed infestation is heavy, serious yield penalties may be avoided by incorporating resistance to triazine herbicides into such hybrids. In Canada alone, 400,000 ha of potentially useful land are infested with wild mustard (*Sinapis alba*) and stinkweed (*Thalspi arvensis*).

Recently, a method has been proposed which would allow single-cross hybrid seed to be economically produced by application of triazine herbicides to field plantings composed of a mixture of triazine-sensitive pollinators and triazine-resistant female plants (Beversdorf et al. 1985). The major biological input required to produce triazine-resistant single-cross hybrid *B. napus* by this method is a female parent that combines cytoplasmic male sterility (cms) and cytoplasmic resistance to triazine herbicides (ctr). These two traits have been closely associated with the organelles, cms with the mitochondria and triazine resistance with the chloroplasts. Such a cytoplasmic combination cannot be produced by conventional breeding methods as the organelles are maternally inherited. However, Pelletier et al. (1983) combined Ogu cms mitochondria with chloroplasts from either *B. napus* or triazine resistant *B. campestris* via protoplast fusion.

Triazine resistance arose spontaneously in a wild population of *B. campestris* growing in competition with maize (Matais and Bouchard 1978). Cytoplasms bearing this tolerance are now available in *B. napus* (Beversdorf et al. 1980). Several *Brassica* cms systems exist, including Nap (Thompson 1972; Shiga and Baba 1973), Ogu (Bannerot et al. 1977) and Polima (Sernyk 1983) types. Among these, the Polima cytoplasm is a good candidate for use in hybrid production because it is available in several *B. napus* cultivars. In addition, fertility restorer lines have been developed (Fan et al. 1986) and most current varieties will serve as sterility maintainers.

In this report we describe two cybrid rapeseed plants carrying Polima cms and triazine resistance. The results were produced from protoplast fusions carried out at two institutions involved in joint research. Although the methodologies employed were different the desired cybrids were obtained by both groups.

The determining factor in choosing a nuclear genotype for the synthesis of an alloplasmic ctr/Polima cms line by protoplast fusion is the regeneration capability of protoplasts from that genotype. It is also preferable that the genotype be a variety in commercial production. For these reasons the widely grown spring-planted Canadian varieties 'Regent' and 'Triton' were chosen. Protoplasts from both varieties are amenable to culture using the techniques described by Barsby et al. (1986) and Chuong et al. (1985).

In order for protoplast fusion to be useful as a plant breeding tool, the technologies involved should be efficient, reproducible, and well controlled. Several methods for selecting in vitro for protoplast fusion products have been developed including one for *Nicotiana* species that employs complementation between gamma-irradiated and metabolically poisoned cells (Sidorov et al. 1981). A similar procedure for selecting *B. napus* cybrids is described in the present report. This method does not require pre-existing mutations, such as auxotrophy or

albinism, in one of the fusion partners, and eliminates the necessity for screening large populations of regenerants to recover the desired cybrid. The method described here utilizes irradiated nurse cells to recover colonies after protoplast fusion.

The cybrids resulting from these experiments were back-crossed to commercial *B. napus* cultivars and the progeny evaluated for field tolerance to triazine herbicides.

Materials and methods

Plant material

Seeds of *Brassica napus* (2n = 4x = 38) Polima-'Regent' and ctr-'Regent' were supplied by the Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada. Triazine-resistant 'Triton' seed was obtained from the Crop Science Department, University of Guelph, Guelph, Ontario, Canada.

Isolation of protoplasts

a) Dark-grown hypocotyls (Polima-'Regent' and 'Triton'). Protoplasts were prepared from dark-grown hypocotyls of Polima-'Regent' by overnight (16 h) incubation in enzyme solution followed by sieving through cheesecloth and flotation in sucrose. For details refer to Barsby et al. (1986).

Protoplasts from 'Triton' hypocotyls were obtained by overnight incubation in enzyme solution followed by sieving through 60 and 44 μm mesh and pelleting by centrifugation at 100 g for 3 min. Details were given in Chuong et al. (1985).

b) Leaf mesophyll (ctr-'Regent' and Polima-'Regent'). Plants of ctr-'Regent' were maintained in growth chambers with a 12 h photoperiod of 160 $\mu Em^{-2}\ s^{-1}$ of photosynthetically active radiation and constant temperature (23 °C). Third and fourth leaves from three week-old plants were surface sterilized by immersion in a 10% solution of 5% commercial preparation of hypochlorite for 3 min, rinsing once in distilled deionised water and once in 70% ethanol. The lower epidermis was brushed gently. The leaves were chopped into 1 cm pieces, incubated overnight in a solution containing 0.1% Cellulase R-10 (Yakult Honsha Co., Ltd., Japan) and 0.01% Macerozyme R-10 (Kinki Yakult Mfg. Co., Ltd., Japan) and protoplasts were recovered by sucrose flotation as described by Barsby et al. (1986).

Polima-'Regent' plants were grown in a growth room with a 16 h photoperiod of 200 $\mu Em^{-2}\ s^{-1}$ of photosynthetically active radiation at the bench surface and at 25/20 °C for light/dark periods. Leaf blades from fully expanded leaves were sterilized with 70% ethanol for 5 s, then a 20 % solution of a 5% commercial preparation of hypochlorite for 10 min, followed by several washes in autoclaved water. The lower epidermis was peeled and the leaf was incubated overnight in an enzyme solution containing 1.5% Cellulase R-10, 0.3% Macerase (Calbiochem), 400 mg/l ampicillin, 10 mg/l gentamycin, 10 mg/l tetracycline and 10% mannitol in CPW salts (Xu et al. 1982). Protoplasts were recovered by centrifugation after sieving the digested leaf material through 60 μm and 44 μm mesh.

Pre-fusion treatments

a) Inactivation of Polima-'Regent' with Iodoacetic Acid (IOA). After isolation, Polima-'Regent' hypocotyl protoplasts were incubated in a solution containing 4 mM IOA and 0.35 M sucrose for 20 min at room temperature. Subsequently, they were centrifuged at 80 g for 5 min and resuspended in 0.35 M sucrose. A second centrifugation (200 g, 10 min) was employed to collect the floating protoplasts.

b) Gamma-irradiation. Ctr-'Regent' mesophyll protoplasts to be used for fusions with IOA-treated Polima-'Regent' hypocotyl protoplasts were subjected to 30 krad gamma-irradiation from a Caesium 35 source.

Protoplast fusion treatments

Iodoacetate-treated hypocotyl protoplasts and gamma-irradiated leaf protoplasts were mixed in a 1 : 1 ratio in test tubes and fused by adding an equal volume of a 25% polyethylene glycol (PEG 8000, Sigma Chem Co., USA) solution to the protoplast mixture. After 10 min the PEG solution was slowly diluted with a 50 mM solution of calcium ions at pH 10.5 (for detailed procedure refer to Barsby et al. 1984).

Untreated Polima-'Regent' mesophyll protoplasts and 'Triton' hypocotyl protoplasts were fused by placing 0.2 ml of the protoplast mixture containing equal numbers of each at 10^6 cells/ml between two 0.2 ml aliquots of a 30% polyethylene glycol solution (PEG 6000, Fisher Scientific) containing 30 mM $CaCl_2 \cdot 2\ H_2O$ and 9% mannitol. After 15 min at room temperature the fusion mixture was diluted gradually with 2.5 ml protoplast culture medium (see below).

Protoplast culture and plant regeneration

Fusion mixtures containing IOA-treated hypocotyl protoplasts and gamma-irradiated leaf protoplasts were cultured according to Barsby et al. (1986) with the modification that a 'nurse culture' of gamma-irradiated (20 krad) *Nicotiana tabacum* mesophyll protoplasts was added. The nurse cells, which were incapable of sustained division, were added to the *Brassica* protoplasts to give a total plating density of 100,000 protoplasts per ml. Subsequent protoplast culture and plant regeneration procedures were performed as described by Barsby et al. (1986). Plants regenerated from fusion experiments were returned to the growth chamber for analysis. To induce flowering the photoperiod was increased to 16 h.

Protoplast fusion mixtures containing untreated 'Triton' hypocotyl protoplasts and Polima-'Regent' leaf protoplasts were cultured at 25 °C in the dark in liquid N20 medium, which is based on the protoplast culture medium described by Chuong et al. (1985) with the following modifications: 4 mg/ml instead of 5 mg/ml of Ficoll 400 and 0.25 mg/l instead of 0.5 mg/l of 6-benzylamino purine (BAP) were used, and no naphthaleneacetic acid (NAA) was added. After one month floating microcalli were transferred onto MS medium (Murashige and Skoog 1962) supplemented with 5 mg/l BAP, 0.5 mg/l NAA and 1.5% sucrose. The plantlets that developed on this medium were transferred into peat pellets to induce rooting and returned to the growth room.

Leaf atrazine test

To test the sensitivity to atrazine of the plants regenerated from protoplasts, a paste of atrazine in water (1.47 g 80% Atrazine WP, Ciba Geigy, in 100 ml water) was painted on a mature leaf. Plants carrying triazine-resistant chloroplasts were not damaged by this treatment but the leaves of sensitive plants (and in many cases the whole plant) turned yellow and died within one or two weeks.

Microdensitometry

Root tips were fixed in ethanol : acetic acid (3 : 1) overnight at 4 °C, hydrolyzed in 5 M HCl at room temperature for 45 minutes, stained in Feulgen for 1 h and squashed in 45% acetic

acid. The DNA content of Feulgen-stained nuclei in root tip squashes was measured cytophotometrically with a Zeiss MPV3 scanning microspectrophotometer set at a wavelength of 560 nm. Only recognizable stages of mitosis (metaphase, anaphase and telophase) were cytophotometrically measured and the values were compared to those obtained from plants of known ploidy level.

Cytoplasmic DNA analyses

Chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) was extracted from leaves of regenerated plants as described by Kemble (1987). The DNAs were fragmented with *Eco* RI, electrophoresed on agarose gels and the cytoplasmic genomes classified according to the characteristic fragment pattern produced (Kemble 1987).

Field testing

Cybrids were pollinated with pollen from *B. napus* genotypes which act as maintainers for the Polima cms cytoplasm. Preliminary field evaluations were carried out on the second sexual generation. Seed was sown with a 'Hege' seed drill in 6 m × 1.5 m 6-row plots. Experimental plots were sown alongside triazine-resistant ('Triton') and triazine-sensitive ('Westar') *B. napus* cultivars as controls. Plots were treated with foliar applications of cyanazine (Bladex, Ciba Geigy) at the recommended rate of 1.5 kg/ha.

Results

The primary aim of the present study was to produce *B. napus* lines that carry both cms and ctr for use in a hybrid rapeseed breeding program. Such plants were obtained from somatic fusion experiments performed with *Brassica* leaf and hypocotyl protoplasts. One cybrid was recovered from a fusion between 'Triton' hypocotyl protoplasts and Polima-'Regent' mesophyll protoplasts (Fig. 1 A). Three plantlets were regenerated from this fusion mixture, which was not subjected to a selection procedure, but only the plant later identified as a cybrid developed to maturity. A second plant (Fig. 1 B) obtained from fusion of iodoacetate-treated Polima-'Regent' hypocotyl protoplasts and gamma-irradiated ctr-'Regent' mesophyll protoplasts also had the desired combination of mitochondria and chloroplasts. Five plantlets were regenerated from this fusion; three survived to maturity: the cybrid, a plant with the Polima cytoplasm and one with the ctr cytoplasm.

Cybrid plants were initially identified on the basis of their resistance to atrazine and their flower morphology. Both cybrids were tolerant to a foliar application of the triazine herbicide, atrazine, in a paste form. Flowers and flower parts of the parent plants and cybrid plants are shown in Fig. 2. Both cybrids had flowers with wrinkled sepals, wavy petals, a thickened pistil and severely reduced arrow-shaped anthers that are characteristic of plants with the Polima cytoplasm.

The *Eco* RI restriction endonuclease patterns (Kemble 1987) of cpDNA and mtDNA confirm the hybrid nature of the cytoplasms (Fig. 3). The cybrids contained ctr chloroplast genomes (differentiated from Polima by possessing an additional fragment of 3.3 kb) and Polima mitochondrial genomes (differentiated from ctr by possessing fragments of 5.4 and 5.1 kb but lacking a fragment of 6.8 kb).

Microdensitometric analysis of Feulgen-stained root tip cells revealed that the 'Triton'/Polima-'Regent' cybrid plant had a level of nuclear DNA corresponding to a chromosome number of approximately 76. The ctr-'Regent'/Polima-'Regent' cybrid had a higher DNA content indicating a chromosome number around 95. The cybrid recovered from the 'Triton'/-Polima-'Re-

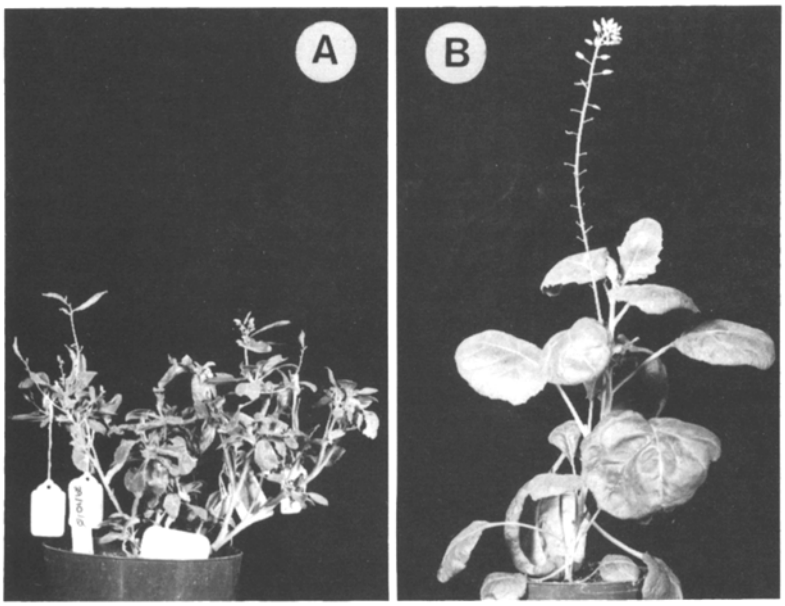


Fig. 1. *B. napus* cybrid plants produced from fusions between **A** Triton hypocotyl protoplasts and Polima-Regent mesophyll protoplasts and **B** IOA-treated Polima-Regent hypocotyl protoplasts and gamma-irradiated ctr-Regent leaf mesophyll protoplasts

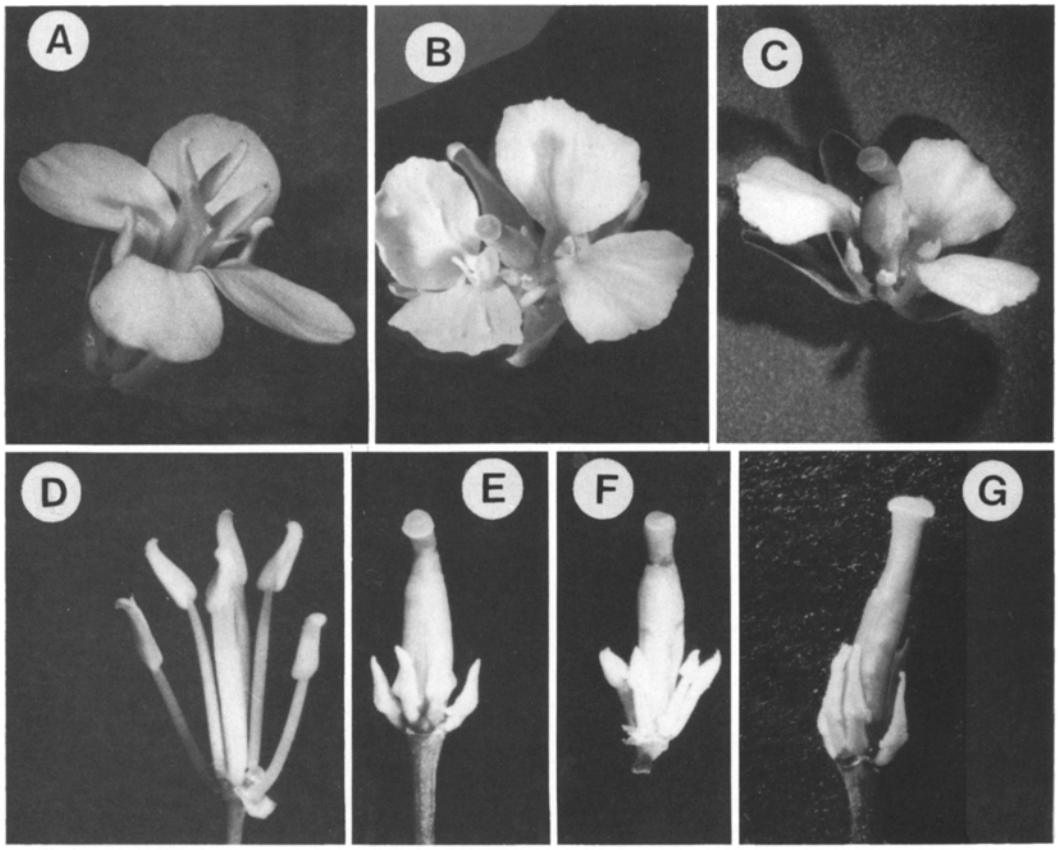
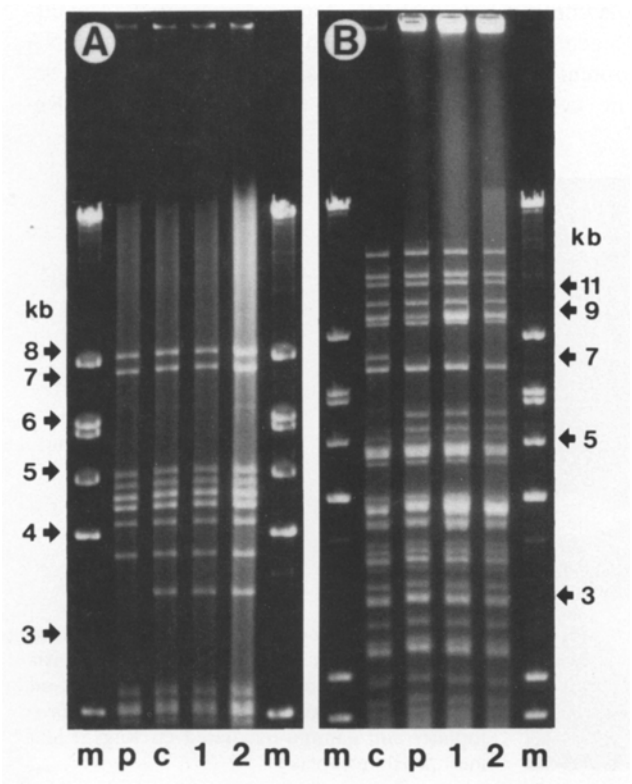


Fig. 2 A–G. Flowers and flower parts of parental lines and cybrid plants. **A** Triton (male fertile) flower, **B** Polima-Regent (male sterile) flower, **C** Triton/Polima-Regent cybrid flower, **D** Triton anthers and pistil, **E** Polima-Regent anthers and pistil, **F** Triton/Polima/Regent cybrid anthers and pistil, **G** ctr-Regent/Polima-Regent cybrid anthers and pistil



gent' fusion was hand-pollinated with pollen of a *B. napus* breeding line and the ctr-'Regent'/Polima 'Regent' cybrid with pollen from 'Regent'. Only a limited amount of seed was produced in both cases, presumably because of the elevated ploidy of the plants. However, viable seed from both cybrids germinated to give morphologically normal plants (Fig. 4 A) with characteristic Polima flowers. Some of these plants were determined to possess a normal allotetraploid chromosome complement ($2n = 4x = 38$) by microdensitometric analysis of Feulgen-stained root tip cells and readily set seed when crossed with a variety of commercial rapeseed cultivars (Fig. 4 B). Cytoplasmic DNA analysis of the seed generation indicated that the combinations of Polima cms mitochondria and ctr chloroplasts were faithfully maternally inherited.

Fig. 3. *Eco* RI restriction enzyme analyses on 1% agarose gels of **A** cpDNAs and **B** mtDNAs. Lane (p) standard Polima pattern expressed by Polima-Regent, (c) standard ctr pattern expressed by ctr-Regent and Triton, (1) ctr-Regent/Polima-Regent cybrid, (2) Triton/Polima-Regent cybrid, (m) size marker fragments produced by independent digestion of lambda DNA with *Eco* RI and *Hae* III

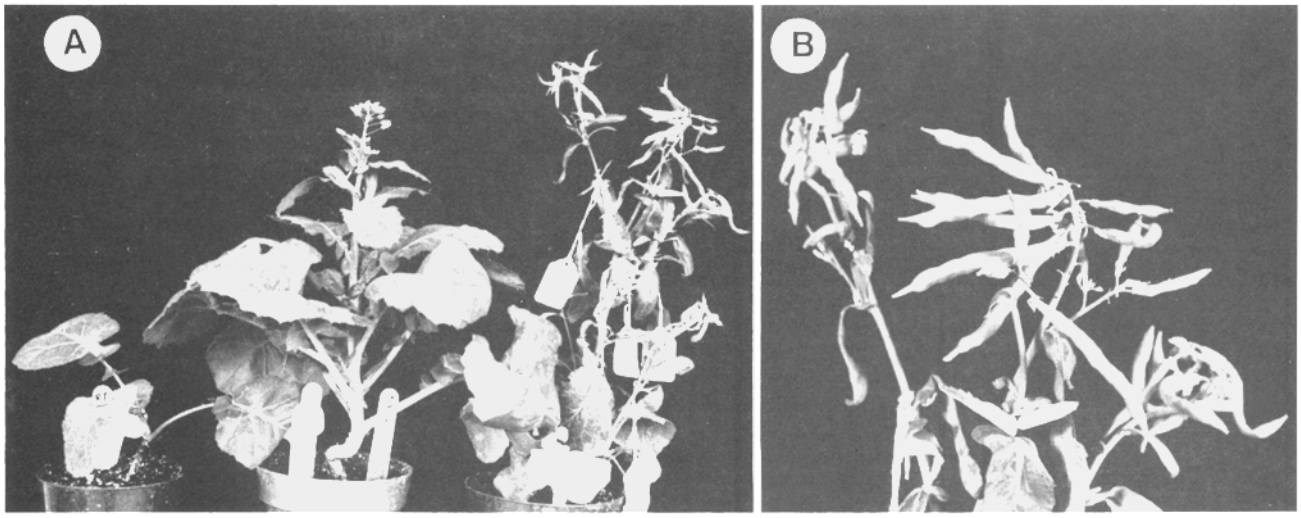


Fig. 4 A, B. Plants grown from seed set on the Triton/Polima-Regent cybrid: **A** seed generation plants at various stages of maturity, **B** full racemes on a seed generation plant

In the field, cybrid progeny and 'Triton' plants were unaffected by triazine application, whereas 'Westar' plants suffered severe chlorosis and stunting. Plants in the field appeared morphologically normal, having characteristic Polima flowers which were visited by insect pollinators. Seed resulted, presumably from pollinations involving pollen from neighboring plots. Seed set appeared normal, but no yield data were taken.

Discussion

The co-segregation of Polima flower morphology and Polima mtDNA demonstrates that Polima cms, like Ogu (Pelletier et al. 1983) is associated with the mitochondrial genome. Moreover, the recovery of a triazine-resistant Polima cms cybrid from the fusion experiment between 'Triton' hypocotyl and Polima-'Regent' leaf protoplasts indicates that the proplastids contributed by the hypocotyl can in some instances outcompete the chloroplasts, from the leaf cell, during the organelle segregation that occurs to establish the cybrid.

This study confirms that direct selection for organellar traits, such as the inclusion of atrazine in the culture medium, is not necessary for the efficient recovery of plants possessing those traits. Treatment of fusion partners with iodoacetate and gamma-irradiation (essentially as described by Sidorov et al. 1981) consistently results in the recovery of relatively small numbers of plants to which a conclusive cytoplasmic DNA screen may be applied. However, when no such treatments are applied, large numbers of plants can result and screening at the plant level becomes impractical (unpublished observations). The question of the agronomic quality of the recovered material has not been addressed in terms of yield, but the treatments applied in culture did not induce any obvious undesirable agronomic changes in the progeny of the regenerated plants. On the contrary, plants in the field were pollinated normally and expressed field tolerance to triazines. Furthermore, the successful recovery of normal seed generations from the cybrids suggests that the novel cytoplasms created here by protoplast fusion may be backcrossed into any chosen *B. napus* spring or winter-planted variety. This seed generation will be crossed further with maintainer lines for seed increase and the progeny will be evaluated as female parents for hybrid seed production.

Thus we have demonstrated that cybrids with important agronomic traits like cms and cytoplasmic herbicide resistance can be recovered efficiently enough to make protoplast fusion a useful technique for incorporation into a plant breeding program. The major stumbling block in the transfer of this technology to other crop species remains the development of systems for the regeneration of plants from protoplasts.

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