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Production and characterization of asymmetric somatic hybrids between *Arabidopsis thaliana* and *Brassica napus*

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Summary. Cell suspension-derived protoplasts of a chlorsulfuron-resistant (GH50) strain of *Arabidopsis thaliana* cv Columbia were X-irradiated at 60 or 90 krad, to facilitate the elimination of GH50 donor chromosomes in fusion products. Irradiated GH50 protoplasts were fused, with polyethylene glycol, to protoplasts derived from stem epidermal strips of *Brassica napus* cv Westar. Chlorsulfuron-resistant colonies were selected in vitro and then transferred to shoot and root regeneration medium. Seventeen hybrid lines were regenerated in vitro, and eight were successfully established in the greenhouse, where they flowered. These eight asymmetric hybrids were intermediate in vegetative morphology between *Arabidopsis* and *Brassica*. The flowers from these hybrids were male-sterile with abnormal petal and pistil structures. Zymograms for phosphoglucomutase, esterase, and peroxidase showed the presence of all parental isozymes in each of the hybrids tested. Nuclear hybridity was also confirmed for the ribosomal RNA genes using a wheat rDNA probe; however, the chloroplast genome in each of the hybrids was derived solely from the *Brassica* parent. All selected somatic hybrids were capable of rooting at levels of chlorsulfuron which were inhibitory to unfused *Brassica* plantlets. The degree of herbicide resistance in the hybrid shoots is presently being evaluated.

Key words: Asymmetric protoplast fusion – "*Arabidobrassica/Brassidopsis*" – Herbicidce tolerance – Gene transfer

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Introduction

The production of intergeneric and interspecific hybrid plants by sexual crossing is often prevented by physical and/or biologial barriers to the reproductive process. With the advent of protoplast culture and fusion technology, these sexual incompatibilities can in some cases be bypassed, so that the production of somatic hybrids from distantly related parents has become a reality (Bonnett and Glimelius 1990).

Protoplast fusion offers the unique possibility of combining unrelated nuclear and cytoplasmic genomes thereby producing hybrid cells and, ultimately, hybrid plants. The maintenance of two unrelated genomes in a hybrid is not always desired since this condition has been associated with morphological and cytogenetic abnormalities often resulting in partial or complete sterility (Gleba and Hoffmann 1980). To produce viable and fertile hybrids, researchers have irradiated protoplasts from one parental line (the donor) in order to pulverize the chromosomes and facilitate the elimination of substantial amounts of the donor genome (Gleba et al. 1988). In this manner, a specific trait native to the irradiated donor genome may eventually be transferred into the genome of the recipient. As this is accomplished without the conservation of the entire donor genome, the term 'asymmetric somatic hybridization' has been applied to such hybridization studies.

A number of asymmetric hybridization studies have been performed in the family *Brassicaceae* . The asymmetric nature of these hybridization products, such as *Brassica campestris* + *Brassica oleracea* hybrids, has been verified using isozyme analysis, chromosome analysis (Yamashita et al. 1989), and in-situ hybridization with species-specific DNA probes (Itoh et al. 1991). Morphogenic potential and fertility have also been increased

Table 1. *Brassica* + *Arabidopsis* hybrids characterized

Hybrid line	Irradiation[a]	Selection		Plants[d]	Flower colour	Rooting[e]
		Level[b]	Day[c]			
1:Ai	90	0.001	0	Y	Light Yellow	Y
1:Aii	90	0.001	0	Y	Light Yellow	N
1:Aiii	90	0.001	0	Y	Light Yellow	Na
1:Aiv	90	0.001	0	Y	Light Yellow	Na
1:Bi	60	0.001	0	Y	Na	Na
1:Bii	60	0.001	0	Y	Na	Na
1:C	60	0.001	5	Y	White-green	
2:A	60	0.01	0	Y	Na	Y
2:Bi	60	0.01	0	Y	Yellow-green	Na
2:Bii	60	0.01	0	Y	Light Yellow	Y
2:C	60	0.01	0	Y	Na	Na
3:A	60	0.01	14	Y	Na	Na
3:B	60	0.01	14	Y	Yellow	Y
3:C	60	0.01	5	Y	White	Na
4:A	0	0.1	5	N	Na	Na
4:B	0	0.1	0	N	Na	Na
4:C	90	0.1	5	N	Na	Na

[a] Total amount of X-rays (Krads) received by *Arabidopsis* protoplasts prior to fusion
[b] Concentration of chlorsulfuron (μg/ml) in the protoplast culture and shoot regeneration media
[c] Number of days following the fusion when selection was applied. Day 0 is day of fusion
[d] Regenerated plants transferred to soil Y (yes), N (no)
[e] The ability of hybrid shoots to root in vitro on medium containing 0.01 μg/ml of chlorsulfuron was scored. Y (yes); N (no); Na, data not available

through irradiation of one fusion partner, as was shown in *Brassica juncea* + *Eruca sativa* hybrids (Sikdar et al. 1990). Symmetric somatic hybrids of *B. campestris* (2n = 20) + *Arabidopsis thaliana* (2n = 10), produced by Gleba and Hoffmann in the late 1970s, were shown to have morphogenic difficulties, infertility, and an inability to establish themselves in soil (Gleba and Hoffmann 1979, 1980). In the present asymmetric hybridization study, *Brassica napus* (2n = 38) was fused to an irradiated *A. thaliana* partner in an effort to isolate hybrids expressing fewer of the abnormal features observed in previous *Brassica* and *Arabidopsis* intergeneric hybrids (Gleba and Hoffmann 1978, 1979, 1980).

We have attempted to regenerate somatic hybrids between *A. thaliana* and *B. napus* for the purpose of gene transfer between these distantly related crucifers. *A. thaliana* serves as an ideal donor species for such a study due to the extensive molecular characterization of its small genome and the availability of many mutants. As a result of its size and simplicity, considerable effort has been put into mapping and cloning the *Arabidopsis* genome, resulting in the characterization of numerous mutant and wild-type genes. One such mutant is the acetolactate synthase gene conferring resistance to sulfonylurea herbicides (Haughn and Somerville 1986). *B. napus* serves as an ideal recipient species for this work since efficient protoplast regeneration protocols have been established for it (Klimaszewska and Keller 1987) and it is known to be sensitive to the sulfonylurea herbicides.

Materials and methods

Plant material

The donor tissue was a leaf callus-derived cell suspension line of *A. thaliana* cv Columbia strain GH50 established by Gleddie (1989). This strain carries a point mutation in the acetolactate synthase gene (Haughn and Somerville 1986) which confers a selectable resistance to sulfonylurea and imidazolinone herbicides. The suspension was maintained by weekly subculture into 40 ml of an MS salt solution (Murashige and Skoog 1962) with B_5 vitamins (Gamborg et al. 1968), 2% sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and kept on a gyratory shaker at 24 °C with a 16-h photoperiod (Gleddie 1989). The recipient fusion partner was *B. napus* cv Westar, grown at 25 °C in the greenhouse with a supplementary fluorescent-incandescent light source having a 16-h photoperiod.

Protoplast isolation and irradiation

Protoplast from the *A. thaliana* cv Columbia GH50 cell suspension were isolated by digesting 0.5 g of filtered cells in 18 ml of

1% macerozyme and 1% cellulase R-10 (Yakult Honsha Co. Ltd., Tokyo, Japan) in MES buffer (5 mM 2-N-morpholinoethanesulfonic acid, 0.4 M mannitol, pH 5.8) for 18 h. Digestion was carried out on a gyratory shaker (100 rpm) at 25 °C in the dark, for 16 h. Petri dishes containing GH50 protoplasts in enzyme solution were irradiated with a Picker Scanray X-ray tube (AC-128 Automatic) 5.0 cm from the source, at 0, 60 or 90 krad. Irradiated protoplasts were filtered through a 44 μm nylon mesh and pelleted by centrifugation at 50 g for 10 min. Protoplasts were re-pelleted in MES buffer and then resuspended in Wash Medium (0.2% $CaCl_2$, 2.5% KCl, pH 6.9) prior to fusion. Westar protoplasts were isolated from stem epidermal and subepidermal strips as described by Klimaszewska and Keller (1987) and resuspended in Wash Medium.

Fusion, protoplast culture and selection

The protoplast fusions were carried out using 25% (w/v) polyethylene glycol (PEG) (8000 MW, Sigma) and 3% (w/v) $CaCl_2$, pH 6.9. Westar and GH50 protoplasts (1×10^6 each) were combined, pelleted, and fused at room temperature in 0.5 ml of PEG solution for 15 min. The PEG was then slowly diluted with an 0.4 M mannitol solution (pH 5.8) to a final volume of 12 ml. Fusion products were gently centrifuged (50 g) and resuspended in L(b) protoplast culture medium (Klimaszewska and Keller 1987) after which 1.5 ml of suspension was plated over a 2-ml 0.6% (w/v) agarose sublayer. Plating densities were 5.0×10^5 protoplasts per ml. Fusion products, along with control petri dishes, were incubated in the dark at 28 °C. On day 7, dishes were transferred to a low light intensity (10 $\mu Em^{-2}\ sec^{-1}$), 25 °C, and finally transferred to a higher light intensity (50 $\mu Em^{-2}\ sec^{-1}$) on day 10.

Protoplast cultures were fed with 1 ml of C medium at day 14 and with 1 ml of D medium at day 21, as described by Klimaszewska and Keller (1987). Selection occurred at day 0, day 5 or day 14 post-fusion. Chlorsulfuron (Glean™), a sulfonylurea herbicide produced by DuPont, was dissolved in phosphate buffer and aseptically added to the molten agarose sublayer at 0.001 μg/ml, 0.01 μg/ml or at 0.1 μg/ml. Controls, for each of the four fusion experiments, included homofusions of each parent as well as unfused protoplast cultures of each parent. Controls were cultured and selected in the same manner as the fusion products.

Plant regeneration from fusion products

After 30 days, colonies ranging from 1- to 2-mm in diameter were transferred to non-selective shoot regeneration medium [SRM (MS-B_5 salts, 3 mg/l zeatin, 1% w/v sucrose, 0.1 M mannitol, 0.6% agarose, pH 5.8)]. Colonies were subcultured monthly until they produced shoots. Shoots, in turn, were transferred to root regeneration medium [RRM (2% sucrose w/v, 0.1 mg/ml NAA, 0.35% w/v gelrite in MS-B_5 salt solution)] and cultured under the same growth conditions as previously mentioned. Rooted plantlets were carefully dislodged from the gelrite and transferred into a soiless mix in a high humidity mist chamber for 1 week prior to being transferred to soil and maintained in the greenhouse.

Confirmation of the hybrid nature of asymmetric somatic regenerants

Morphological analysis. The flower morphology of greenhouse-acclimatized regenerants was assessed visually by examining at least ten individual flowers per hybrid, under a dissecting microscope. The nature of the upper leaf surface of these greenhouse hybrids was compared to the upper leaf surface of the parents using Scanning Electron Microscopy (SEM). Freshly excised leaves were immediately placed onto the cryostage of the electron microscope (Hexland Cryotrans CT 1000). The leaf samples were cooled to −80 °C in order to sublime any condensation, and then coated with gold. Samples were cooled again to −150 °C and placed in the path of the electron beam of an AMR 1000 A SEM.

Fig. 1 a, b. Somatic hybrid morphology. **a** Greenhouse-grown "Brassidopsis" hybrid line 2:1A between the parents *A. thaliana* (*1*) and *B. napus* (*3*). **b** Note the intermediate vegetative morphology, large number of flowers, and branching habit of this hybrid

Isozyme analysis. Leaf or root tissue was carefully harvested from young (1 to 2 months-old) greenhouse-hardened regenerants and their parents. Protein extraction buffer consisted of 30 mM Dithiothreitol (DTT; J. T. Baker) in 0.2 M Tris-HCl, pH 8.5. Tissue was ground with seasand, followed by centrifugation (12,000 g, 15 min), and the total protein concentration was determined with a BioRad protein assay. Samples consisting of 15 μg of protein and 15% (v/v) loading dye (50% sucrose, 0.1% bromophenol blue) were loaded into the wells of a vertical non-denaturing 6.25% polyacrylamide gel (BioRad Protean II). Samples were electrophoresed at 200 V (constant), and the separating gels were either stained for esterase (EST) (Wetter and Dyck

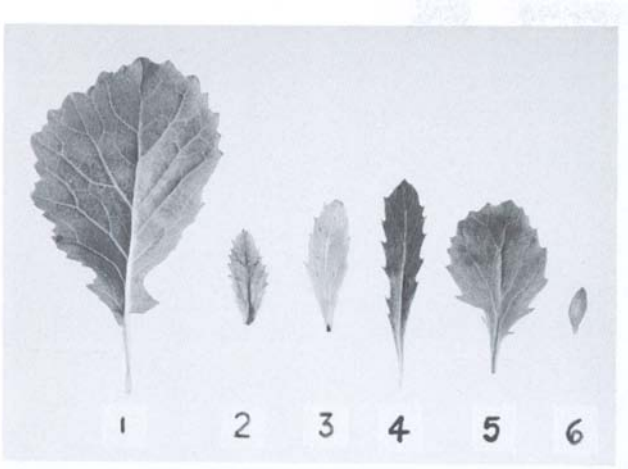

Fig. 2. Leaf morphology of the *B. napus* + *A. thaliana* hybrids. Leaves of hybrids 1:Ai (*2*), 1:C (*3*), and 2:A (*4*), bear a greater resemblance to *Arabidopsis* leaves (*6*), while hybrid 3:B (*5*) exhibits a greater resemblance to *Brassica* leaves (*1*)

1983), phosphoglucomutase (PGM) (Thorpe et al. 1987), or peroxidase (PER) activity, according to the manufacturer's specifications (Schleicher and Schuell).

Southern hybridizations. Genomic DNA, extracted from the leaf tissue of the parents and of the hybrids (Dellaporta et al. 1984), was subjected to restriction enzyme digestion using *Eco*RI, *Bam*HI, *Pst*1, or *Xba*1, followed by electrophoresis and Southern blotting (Southern 1975) to Nytran nylon membranes (Schleicher and Schuell). These filters were probed by DNA hybridization to radiolabelled (by random priners), cloned *Nicotiana* chloroplast fragments (Aviv et al. 1984). Nuclear DNA was analyzed by hybridization of total genomic digests to non-radioactively labelled [by random primed DNA-labeling with digoxigenin – dUTP and lumigen PPD detection (Boehringer, Mannheim)] rDNA sequences cloned in pTA71 (Gerlach and Bedbrook 1979).

Results

A total of four fusion experiments were performed. In experiments 2, 3 and 4, the initial chlorsulfuron selection level of the fusion products was increased from 0.001 µg/ml to 0.01 or 0.1 µg/ml, due to the presence of several *Brassica* escapes at the lower selection level. Surviving colonies were transferred from selection medium to SRM medium where 4% of the fusion product calli, selected at 0.001 µg/ml of chlorsulfuron, regenerated shoots, while 0.9% of those calli selected at 0.1 µg/ml chlorsulfuron, produced shoots. Of the unselected *Brassica* control calli, 14.5% produced shoots after 30 days. In many cases, the regenerated shoots were vitrified and required many in-vitro passages before they developed normally. Shoots could then be transferred to RRM where most developed roots.

A total of 17 hybrid plants, originating from either 0, 60 or 90 krad treatments, were regenerated from these

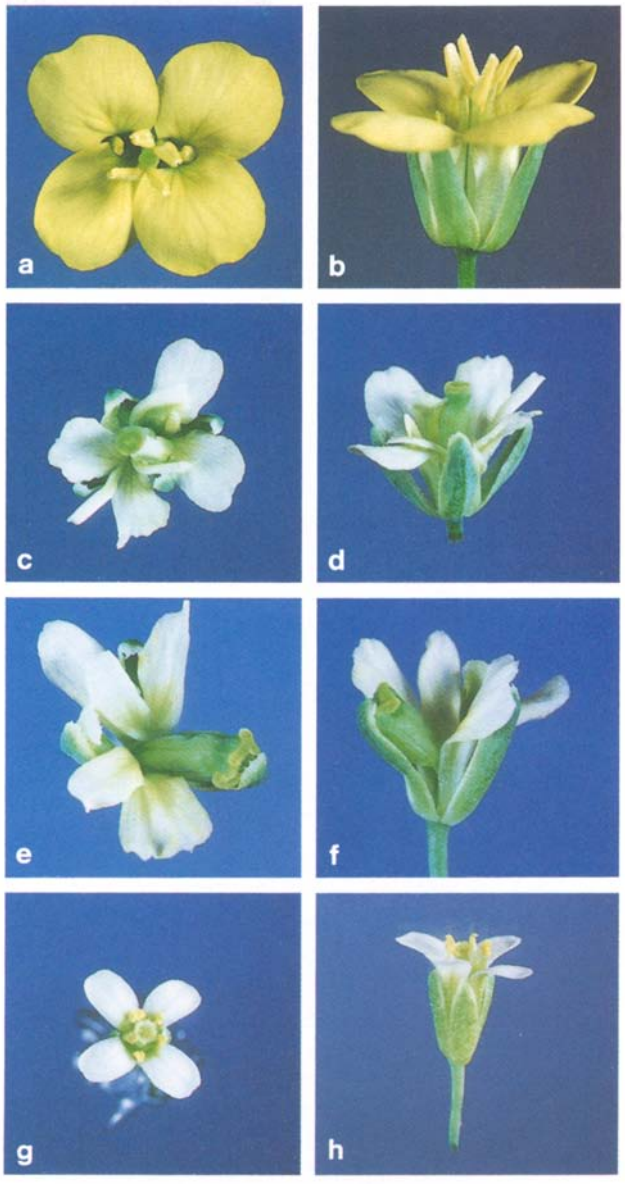

Fig. 3 a–h. Floral morphology of *Brassica, Arabidopsis,* and hybrid plants. *Brassica* flowers (**a, b**) are dark yellow and larger than *Arabidopsis* flowers (**g, h**). Somatic hybrid plants 3:C (**c, d**) and 1:C (**e, f**) display floral abnormalities common to most of the hybrids, with long filamentous anthers (**c**), lack of anthers (**e**), and curved styles (**f**)

experiments (Table 1). High dosage X-ray treatments, such as 60 and 90 krad, were chosen since lower X-ray levels were less effective in arresting the division rate and colony formation of *Arabidopsis* protoplasts. The *Arabidopsis* protoplast-derived callus which as produced, however, could not regenerate shoots on the shoot regeneration medium used in this study.

Each of the hybrids possessed morphologial or biochemical features which distinguished them from the parental species. Only eight hybrid lines were successfully

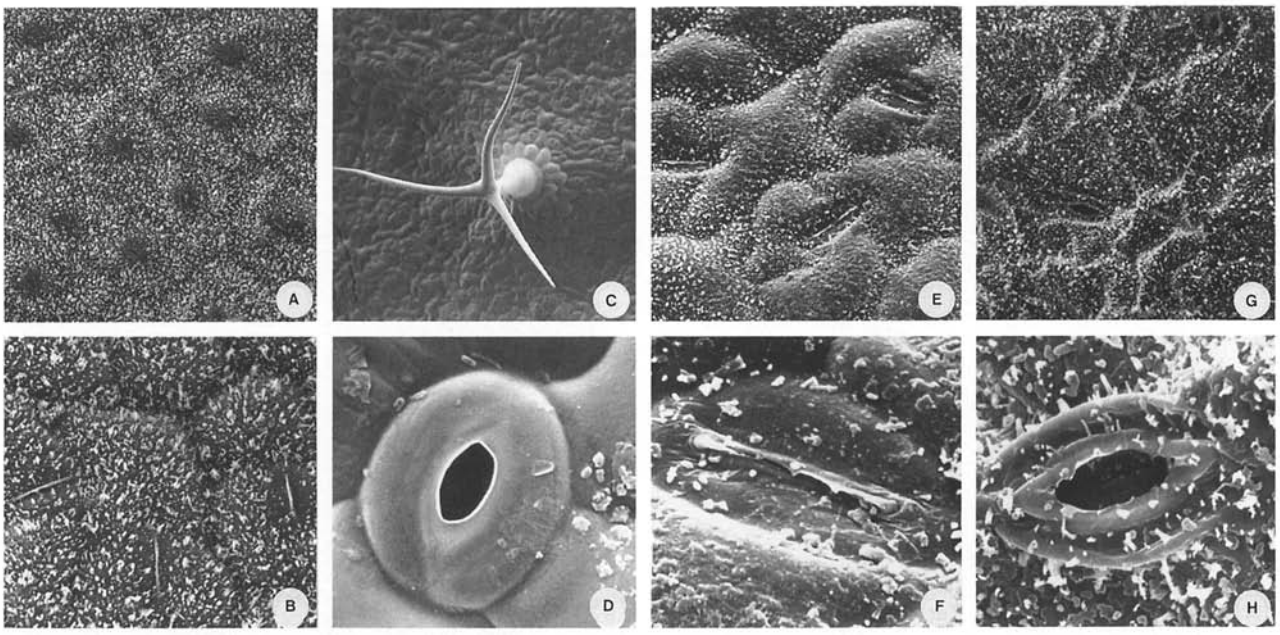

Fig. 4 A–H. Scanning electron micrographs of the upper leaf surfaces of somatic hybrids and parental plants. **A** and **B**: *B. napus* cv Westar (**A**, 500×; **B**, 1,000×). Note the granular wax deposits and single-branched trichomes in **B**. **C** and **D**: *A. thaliana* cv Columbia (**C**, 100×; **D**, 3,000×). Note the tri-branched trichomes, and smooth leaf surface. **E** and **F**: Somatic hybrid 1:Aiii (**E**, 500×; **F**, 3,000×). **G** and **H**: Somatic hybrid 1:Aiv (**G**, 500×; **F**, 3,000×). Note the waxy leaves of the hybrids and the complete absence of trichomes

established in the greenhouse, where they flowered and developed reduced, curled and shrivelled siliques. All of the hybrid plants were intermediate in size to the parents (Fig. 1). The leaves also reflected this intermediate hybrid shape and size (Fig. 2). The corollas of the hybrid flowers, which ranged from 4.5 to 7.0 mm, were median in length to the corollas of the two parents (Fig. 3). Flowers from *B. napus* plants grown from seed had corolla lengths ranging from 12 to 14 mm, while flowers from *Brassica* plants regenerated from protoplasts possessed corolla lengths of 14–15 mm. Corollas from seed-grown *A. thaliana* cv Columbia flowers were always in the range of 2.0–2.5 mm in length. The hybrid petal colour varied from yellow to white-green in various regenerants (Table 1, Fig. 3), while the number of petal-like structures ranged from six to eight. The flowers of both parents had four petals. All of the hybrids possessed four intermediate-sized yet morphologically normal sepals, but all lacked differentiated anthers. Most regenerants, except hybrids 2:A and 3:B, displayed abnormal female reproductive structures such as bent stigmas and multiple carpel-like structures (Fig. 3). A variation in the expression of these abnormal traits was occasionally observed between flowers on the same hybrid plant.

The morphology of the upper leaf surfaces of the greenhouse-hardened hybrids was more similar to that of the *Brassica* parent than to the *Arabidopsis* parent. Of the four "Brassidopsis" plants examined by SEM, none had trichomes, and all exhibited a thick waxy cuticle, like *B. napus* (Fig. 4).

The level of chlorsulfuron resistance expressed by each of the hybrids is being assessed both in vitro, in vivo and by enzyme assays (Bauer-Weston et al., in preparation). Most of the hybrids tested were able to produce roots in vitro after 2 weeks of culture on 0.01 μg/ml of chlorsulfuron (Table 1). *B. napus* shoots do not root in vitro on RRM supplemented with 0.01 μg/ml of chlorsulfuron and only 10% of these shoots regenerated roots on 0.001 μg/ml of chlorsulfuron.

Hybridity was investigated at the biochemical level through isoenzyme analyses using PER, EST and PGM enzyme activity staining. These three isozymes clearly demonstrated hybrid banding patterns among the regenerants. The PGM zymogram of leaf protein and the EST zymogram of root protein showed *Brassica*-specific and *Arabidopsis*-specific bands in all of the hybrids (data not shown). The PER isozyme study (Fig. 5) was also successful in demonstrating the expression of all parental isozymes in the hybrids. The *Arabidopsis* parent exhibited one intermediate-migrating band and the Westar parent exhibited two slow-migrating bands along with one fast-migrating band. All of the hybrids revealed four peroxidase bands which had the same migration rates as those of the parents.

Nuclear hybridity was also confirmed by probing restriction endonuclease-digested genomic DNA of the hy-

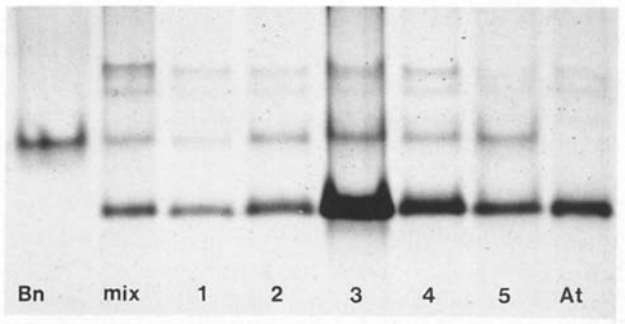

Fig. 5. Electrophoretic separation and peroxidase isozyme activity staining of leaf extracts of *Brassica* (*Bn*), a mixture of *Brassica* and *Arabidopsis* (*mix*), somatic hybrids 1:Ai, 1:Aii, 2:Bi, 3:B, 4:A (*lanes 1–5*), and *Arabidopsis* (*At*)

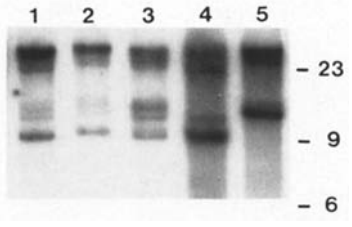

Fig. 6. Southern hybridization of *Pst1*-digested genomic DNA of the parental plants and hybrid plants to a non-radioactively labelled ribosomal DNA probe, pTA71. *Lane 1,* hybrid 3:C; *lane 2*, hybrid 2:A; *lane 3*, hybrid 1:Ai; *lane 4, Brassica*; *lane 5*, *Arabidopsis*. Molecular weight markers in kbp

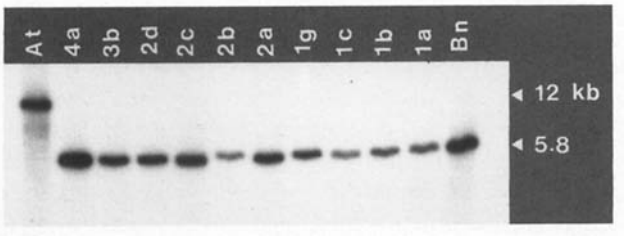

Fig. 7. Southern hybridization of *Xba1*-digested genomic DNA of the parents and hybrids to a radioactively labelled cpDNA probe, pBa1–9. Lanes are identified as: *At, Arabidopsis*; 10 hybrid lines; *Bn, B. napus*. Molecular weight markers in kbp

brids and parents with a heterologous wheat rDNA fragment, pTA 71. The presence of rDNA bands specific to both *Arabidopsis* and *Brassica* was detected in hybrids 2:4, 8:3, and 8:5 (Fig. 6).

The inheritance of chloroplasts among the regenerated "Brassidopsis" plants was investigated by probing genomic DNA from the parents and the hybrids with two different choroplast DNA probes. The first, pBa 1-9, is a tobacco chloroplast DNA probe which can be used to distinguish between *Arabidopsis* and *Brassica* chloroplast DNA. When *Xba*I-digested genomic DNA of the hybrids was probed with pBa 1-9, banding patterns identical to the *Brassica* parent were observed (Fig. 7). A similar result was obtained if genomic DNA of the hybrids was digested with either *Eco*RI or *Bam*HI endonucleases (data not shown), prior to hybridization with this probe. The second chloroplast-specific probe, used in the Southern-hybridization analysis of the "Brassidopsis" hybrids, was total purified chloroplast DNA of *B. napus*. In this case the hybridization pattern also confirmed the inheritance of only *Brassica* chloroplasts among each of the hybrid plants (data not shown).

Discussion

Preliminary investigations demonstrated that high level of irradiation, such as 90 krad, could not be used to inhibit the survival of GH50 protoplasts. Viability, measured by fluorescein diacetate staining, was not dramatically affected by high levels of radiation, nor was division frequency (Bauer-Weston 1990). Although GH50 protoplasts occasionally formed colonies following high-level irradiation, they were not able to regenerate shoots on the regeneration medium (optimized for *B. napus*, Klimaszewska and Keller 1987) used in this study. The regeneration of *Arabidopsis* plants from cell suspensions and protoplasts has been previously shown to be highly dependent upon culture medium composition (Gleddie 1989; Ford 1990). Therefore, prior to fusion, GH50 protoplasts were X-irradiated at 60 or 90 krads for the purpose of genomic destabilization and chromosome elimination from the 'donor' genome.

Of 17 hybrid plantlets regenerated in vitro, eight were successfully rooted and established in soil in the greenhouse. The symmetric hybrids, 4:A and 4:B (Table 1), regenerated shoot structures in vitro, but were never able to develop roots, nor were they ever successfully established in soil. Similar problems were observed in the symmetric "Arabidobrassica" hybrids produced in the late 1970s by Gleba and Hoffmann (1978, 1979). On the other hand, when irradiation was used in the present study, there was some success in establishing eight of the fifteen plantlets in soil. The inability of some of the asymmetric "Brassidopsis" hybrids (named so as to account for the attempted elimination of the *Arabidopsis* genome) to acclimatize to a greenhouse environment may have been due to vitrification. It was observed that highly vitrified plant tissue generally browned and died when placed in a high humidity mist chamber. The presence of extra donor chromosomes (of *Arabidopsis*) may also have had deleterious effects on the regenerant's capability to acclimatize.

From visual observations in the present study, the X-ray pretreatment of the donor protoplasts did not appear to produce fusion products containing self-fertile

flowers, as was hoped. Instead, an array of different flower morphologies was observed (Fig. 3). Most of the hybrid flowers displayed pistils containing a greater number of carpels than in the *Arabidopsis* or *Westar* flowers. Stamens were usually vestigial or absent and sometimes replaced by carpel-like structures. The only unaffected floral structures were the sepals. These characteristics share many similarities with several well characterized homeotic mutations in *Arabidopsis*. The *pistillata* mutant, which has been linked to chromosome 5, has phenotypically been associated with absent stamens, stamens with carpel-like features, and sepals which have replaced petals. Various flower development mutations have also been shown to produce a flower where the perianth is replaced by one whorl of carpel-like organs and the anthers are usually absent (flo4), as well as flowers expressing one or two extra organs per whorl (flo5) (Haughn and Somerville 1988). Variation between flowers on the same plant, as observed in many of these "Brassidopsis" regenerants, may be due to a genetic alteration which is incompletely penetrant, and so vary the expression of floral traits. The cause of the floral aberrations observed in our hybrids cannot be determined from these studies, but may be due to several factors: a combination of two sets of genes directing flower development; an incomplete elimination or inexpression of some flower development genes; or mutations, possibly caused by the X-irradiation of *Arabidopsis*, in some flower-specific genes.

The "Arabidobrassica" hybrids produced by Gleba and Hoffmann, through somatic hybridization, also demonstrated bizarre floral abnormalities such as a lack of anthers or petals, transformed floral structures and a petal colour varying from predominantly white to light-yellow and sectionally yellow. All of these hybrids were self sterile (Gleba and Hoffmann 1978, 1979; Hoffmann and Adachi 1981). Even though these plantlets were not produced by asymmetric hybridization using irradiation to direct chromosome elimination, extensive chromosome recombination and elimination of both parental genomes was observed. The nondirected and random elimination of chromosomes in the hybrid cell line gave rise to the various cell regenerants which these authors termed "asymmetric hybrids", and which were capable of limited morphogenesis into plantlets in vitro but could not be maintained in soil (Hoffmann and Adachi 1981).

In this study, *B. napus* and the hybrids, when grown in vitro, all produced leaf hair structures (trichomes) which were lost after acclimatization in a greenhouse environment. The trichomes produced on in-vitro propagated *Brassica* plants were simple in structure having only a single branch, whereas the *Arabidopsis* trichomes were branched. Even after acclimatization to a greenhouse environment, *Arabidopsis* maintained its tri-branched leaf hair structure (Fig. 4). In the study by Gleba and Hoffmann (1979), only leaves from in-vitro hybrids were examined, and they were often deformed showing no upper or lower surfaces. Some of the in vitro plantlets, though, did demonstrate trichome structures which were intermediate to the tri-branched *Arabidopsis* hairs and the unbranched *B. campestris* parent (Gleba and Hoffmann 1979, 1980).

Five hybrid plants were assayed for PGM, PER, and EST enzyme activities. The expression of all *Arabidopsis* and *Brassica* isozymes by the hybrids was verified for PGM (data not shown) and PER using leaf protein (Fig. 5), and for EST using root protein (data not shown). For these isozyme assays, all of the *Brassica* and *Arabidopsis* alleles appear to be expressed in the hybrid extracts; hence, none were lost through the asymmetric fusion and culture procedure.

The wheat ribosomal DNA probe, pTA 71, is a full length rDNA repeat unit which includes the structural genes for 26 s, 18 s, and 5.8 s ribosomal RNAs as well as spacers. In plant species the number of ribosomal gene repeats is usually in the order of 10^4 per genome, making this probe a very sensitive marker for characterizing nuclear DNA enriched with ribosomal RNA genes (Gerlach and Bedbrook 1979). Due to its strong hybridization to nuclear plant DNA, pTA 71 has been effectively used to diagnose the nuclear origin of interspecific somatic hybrids of *Medicago* (Thomas et al. 1990), tobacco (Sproule et al. 1991), and now crucifers (Fig. 7). Besides demonstrating nuclear hybridity, pTA 71 has also shown the loss of some *Brassica*-specific and *Arabidopsis*-specific rDNA bands in some of the hybrids (data not shown).

Chloroplasts are maternally inherited in most plant species; however, by protoplast fusion the chloroplasts of two different species can exist in a single fused heterokaryon. In the present study, Southern-blot analyses of the "Brassidopsis" plants indicated that their chloroplasts were identical to the *Brassica* parent (Fig. 7). No evidence for chloroplast recombination was observed using either the *Brassica* chloroplast DNA probe or a heterologous tobacco chloroplast DNA probe. In a similar fusion study between two different varieties of *B. napus*, only the chloroplasts from the unirradiated recipient were present in the fusion products (Menczel et al. 1987). Also, in cases of intergeneric fusion, such as *Nicotiana* + *Daucus*, the chloroplast genome of the hybrids was derived solely from the unirradiated recipient (Smith et al. 1989). In some symmetric intergeneric fusion studies, such as *Nicotiana* + *Petunia*, the chloroplasts of the hybrids were inherited only from the *Nicotiana* parent (Pental et al. 1989), while in some interspecific fusions non-random chloroplast segregation has also been observed (Gleddie et al. 1983; Donaldson et al. 1992). It is unlikely that the irradiation treatment of *Arabidopsis* protoplasts in this study was responsible for the elimination of *Arabidopsis* chloroplasts, since fusions of unirradiated *Arabidopsis* protoplasts also resulted in a plant with

Brassica plastids (Fig. 7). This may indicate that chloroplast segregation in somatic hybrids may not only depend on the irradiation of a donor protoplast, but on the species which are being fused, or the state of differentiation of the tissue source from which protoplasts are obtained, or on complex nuclear-cytoplasmic interactions, or upon some combination of these factors. The apparent uniparental inheritance of *Brassica* chloroplasts may simply be coincidental since this sample size of asymmetric and symmetric plants was small.

The "Brassidopsis" somatic hybrids produced in this study all appear to possess nuclear DNA from both parents with no clear evidence for the extent of *Arabidopsis* genome elimination. Such information could only be derived from a detailed cytogenetic analysis of the hybrids. Morphologically, the regenerants demonstrated hybrid characteristics, yet unfortunately they were all male-sterile. The maintenance of only the *Brassica* chloroplast DNA in the hybrids may, or may not, have been influenced by the X-ray treatment since the one unirradiated symmetric somatic hybrid plant also possessed only *Brassica* chloroplasts.

The effectiveness of asymmetric somatic hybridization as a method for introducing a novel trait into a regenerant is dependent upon the gene or genes which are being transferred, and on the ability to select hybrids in vitro. This method has potential use for the transfer of molecularly uncharacterized traits, and for multigenic traits, though one of the limitations of this hybridization system is the lack of control over the amount of DNA transferred from the donor to the recipient cell. This study has shown that herbicide resistance is a trait which can be used to select for asymmetric hybridization in vitro. However, even when a donor species is treated with high levels of X-irradiation, the amount of donor DNA which is lost is still partially dependent upon other factors. This suggests that further investigation into the determinants responsible for nuclear elimination and recombination in somatic cells will be needed in order to exploit this technology to produce morphologically sound and fertile hybrids.

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