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Incorporation of hygromycin resistance in *Brassica nigra* and its transfer to *B. napus* through asymmetric protoplast fusion

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Summary. With the idea to develop a selection system for asymmetric somatic hybrids between oilseed rape (*Brassica napus*) and black mustard (*B. nigra*), the marker gene hygromycin resistance was introduced in this last species by protoplast transformation with the disarmed *Agrobacterium tumefaciens* strain C58 pGV 3850 HPT. The *B. nigra* lines used for transformation had been previously selected for resistance to two important rape pathogens (*Phoma lingam, Plasmodiophora brassicae*). Asymmetric somatic hybrids were obtained through fusion of X-ray irradiated (mitotically inactivated) *B. nigra* protoplasts from transformed lines as donor with intact protoplasts of *B. napus*, using the hygromycin resistance as selection marker for fusion products. The somatic hybrids hitherto obtained expressed both hygromycin phosphotransferase and nopaline synthase genes. Previous experience with other plant species had demonstrated that besides the T-DNA, other genes of the donor genome can be co-transferred. In this way, the produced hybrids constitute a valuable material for studying the possibility to transfer agronomically relevant characters – in our case, diseases resistances – through asymmetric protoplast fusion.

Key words: *Agrobacterium*-transformation – Asymmetric protoplast fusion – *Brassica napus* - *Brassica nigra*

Introduction

One important limitation in the use of cell culture methods for breeding purposes is the difficulty of finding effective in vitro selection systems for genotypes carrying agronomically desirable traits, because generally these traits do not express at cell culture level. The introduction of selectable dominant marker genes in valuable genotypes by transformation techniques should permit a rapid identification in vitro of cells carrying such genotypes under conditions of selection pressure.

We present here the results of *Agrobacterium*-mediated protoplast transformation experiments leading to the introduction of the selectable marker gene, hygromycin phosphotransferase, in some black mustard (*Brassica napus*) gentoypes previously selected for a double disease resistance: to blackleg (*Phoma lingam*) and clubroot (*Plasmodiophora brassicae*). Part of the results of the transformation experiments have been published (Sacristán et al. 1988).

The final aim of this work is the transfer of the mentioned pathogen resistances from black mustard (donor) into oilseed rape (*B. napus*) by asymmetric protoplast fusion, using the hygromycin resistance as selection marker for fusion products. It has been demonstrated that it is possible to transfer marker genes introduced by *Agrobacterium*-transformation from an inactivated donor into recipient protoplasts, and that in this process, besides the T-DNA, other genetic material of the donor genome can be co-transferred (Müller-Gensert and Schieder 1987).

First results on the production of asymmetric somatic hybrids obtained through fusion of X-ray irradiated (mitotically inactivated) *B. nigra* protoplasts with intact protoplasts of *B. napus* are also presented in this paper. They demonstrate the suitability of the selection procedure for an unequivocal recovery of somatic hybrids.

Materials and methods

Plant material

Six *B. nigra* genotypes selected for a double disease resistance (see Results) were used as material for transformation. Their origin is given in Table 1.

Table 1. Origin of *Brassica nigra* lines selected for *Plasmodiophora brassicae* resistance after infection with clubroot phathotype ECD 19/31/31

B. nigra line	Origin
229	B.G. Kew, England
460	Hort. Bot. Hauniensis
461	Orto Bot. Udine, Italy
2051	"Junius", Petersen, FRG
2058	"Junius", Petersen, FRG
16220	Svalöf, Sweden

Transformed, hygromycin-resistant *B. nigra* callus lines served as donor material for experiments of asymmetric protoplast fusion. They were maintained on modified 2N-medium (Chuong et al. 1987) containing 25 mg/l hygromycin B (Boehringer, Mannheim).

B. napus summer rape line H_1 (Sacristán 1981) was used as recipient parent.

Plant material was maintained as sterile shoot cultures on medium containing salts of MS medium (Murashige and Skoog 1962), organics of Gamborg medium (Gamborg et al. 1968) and 0.2 mg/l α-naphthalene acetic acid (NAA).

Agrobacterium strain

The disarmed *Agrobacterium tumefaciens* strain C58 containing the pGV 3850 HPT plasmid was used for transformation. This plasmid carries within the T-DNA the coding region of the hygromycin phosphotransferase gene fused to the nopaline synthase promoter, and confers hygromycin resistance to transformed plant cells (Van den Elzen et al. 1985). The strain was a gift from Prof. L. Willmitzer (Institut für Genbiologische Forschung, Berlin West).

The bacterial strain was maintained on full medium (Difco nutrient broth 8 g/l, sucrose 5 g/l, yeast extract 1 g/l, agar 15 g/l) under selective conditions (100 mg/l rifampicin + 100 mg/l spectinomycin).

Protoplast isolation, co-cultivation and selection

Mesophyll protoplasts were isolated from leaves of sterile shoot cultures by overnight digestion of leaf stripes in 0.05% macerozyme R-10, 0.25% cellulase R-10 and 0.6 *M* mannitol (650–700 mOsm).

Callus protoplasts from transformed *B. nigra* lines were obtained by overnight digestion of 1–2 mm callus pieces in 0.5% macerozyme R-10, 1% cellulase R-10 and 0.6 *M* mannitol.

Protoplast purification was done as described by Schieder (1984) using sea water as wash solution and 0.6 *M* sucrose for floating.

Protoplasts were plated at a density of $10^5 - 2 \times 10^5$ protoplasts/ml in MI medium (Li and Kohlenbach 1982) and cultured first in the dark at 25 °C.

Mesophyll protoplasts of *B. nigra* were isolated as described. Four to six days after isolation, the protoplast cultures were inoculated with agrobacteria at an initial proportion of 100–300 bacteria per protoplast. After 48 h co-cultivation, bacterial growth was inhibited by adding Claforan (Hoechst, Frankfurt) to a final concentration of 500 µg/ml. Four days later, protoplast cultures were embedded in MI-seaplaque-agarose, and the agarose blocks were transferred to plates containing liquid K3 medium (Glimelius 1984), supplemented with 25–35 mg/l hygromycin B for selection (bead-type culture system). The cultures were then transferred from the dark to continuous illumination (1000 lx). The liquid selective medium was replaced weekly by fresh medium. Resistant colonies could be distinguished 4–6 weeks after selection started. Growing colonies sized ≥ 2 mm were individually transferred to MS-agar medium containing 0.625 mg/l of each 6-benzylaminopurine, kinetin and zeatin, 0.5 mg/l NAA, 200 mg/l casein hydrolysate (2N-medium of Chuong et al. 1987, modified) and 25 mg/l hygromycin.

Nopaline synthase assay

Nopaline synthase activity was assayed as described by Otten and Schilperoort (1978). Electrophoresis was carried out at 400 V for 45 min. After staining, the spots were visualized under UV light. In the cases of unclear results, the tissues were cultured ahead of time for 1–2 weeks on medium supplemented with 3×10^{-5} *M* 5-azacytidine and the tests were repeated.

Asymmetric protoplast fusion and selection of somatic hybrids

Protoplasts from transformed *B. nigra* calli were isolated as described, suspended in sea water, plated and immediately exposed to X-rays (X-ray equipment Phillips MGCO1). Irradiation was applied at 90 kV, 12.5 Grays (Gy)/min, to a dose of 200 or 300 Gy.

B. napus mesophyll protoplasts were suspended in sea water and adjusted to the same concentration as *B. nigra*-irradiated protoplasts ($5 \times 10^5 - 10^6$ protoplasts/ml). Protoplast suspensions of both partners were mixed 1:1. Protoplast fusion was performed by using a polyethylene glycol procedure (PEG solution: 40% PEG 6000, 0.1 *M* $Ca(NO_3)_2$, 0,4 *M* mannitol, pH 9). Depending on the yield of callus protoplasts from *B. nigra*, the fusion process was done in centrifuge tubes (0.5 ml protoplast mixture + 0.5 ml PEG solution) or in petri dishes (50 µl-drops), as described by Menczel et al. (1987). After 10–15 min incubation, 6 volumes of 0.275 *M* $Ca(NO_3)_2$, pH 6 or, respectively, 2 volumes of MI medium + 50 m*M* morpholinoethane sulfonic acid (MES), pH 6, were slowly added for a further 15–30 min incubation. After washing, post-fusion protoplasts were resuspended in MI medium, plated at a density of $10^5 - 2 \times 10^5$ ppl/ml and cultured in the dark. Two weeks later, hygromycin to a final concentration of 25 µl/ml was added for the selection of fusion products, and 6–8 days thereafter the cultures were transferred to light. Dilution with 2N liquid medium containing 25 mg/l hygromycin was done every 10 days. Green colonies were already visible 30 days after beginning of the experiment. When they reached a size of 5 mm or more (6–8 weeks after start of the selection), they were transferred to 2N agar medium.

Plant regeneration from fusion products

For inducing morphogenesis, a two-step procedure based on that described by Kirti (1988) was applied. Callus pieces (0.5–1 cm) were transferred from 2N onto first-step medium (R1: MS + 10% coconut water, 3 mg/l 2,4-D), on which they remained 15–20 days. At the end of this period, most cultures developed globular structures (embryoids) which tended to necrotize. Fresh white embryoids were transferred to second-step medium (R2: MS + 10% coconut water, 3 mg/l 6-benzylaminopurine, 0.1 mg/l gibberellic acid), on which immediately or afterwards some passages shoots from single somatic embryos developed. These were transferred to hormone-free medium with reduced sucrose content (10%). Well-developed shoots were then isolated and transferred to Gamborg medium containing 0.2 mg/l NAA for rooting.

Confirmation of the hybrid character by isozyme analysis

Callus and leaf extracts of putative fusion products were electrophoresed on starch gels using a L-histidine/Na-citrate buffer system (pH 5.7) and stained for esterase activity, according to Shields et al. (1983).

Chromosome numbers

The chromosome numbers of regenerated hybrids were determined in root tips of plants grown as shoot cultures on agar and from plants already transferred to soil, as described (Sacristán and Gerdemann 1986). Three to seven plants per clone and three root tips per plant (on average 20 metaphases) were analysed.

Results

Selection of B. nigra donor lines

B. nigra possesses in its B genome genes conferring resistance to *Phoma lingam*, a resistance which is mostly expressed already at the seedling stage. Cases of cotyledonary lesions, after infection with high virulent pathogen isolates, have been described (Sjödin and Glimelius 1988), but a systemic phase in which the fungus spreads from the cotyledons to the stem base, originating the typical blackleg symptoms of the mature plant – as it occurs in rape and other susceptible Brassicas – has been not yet observed in *B. nigra* (M. D. Sacristán, unpublished results; A. M. Chevre, INRA, personal communication). Therefore, only a sample of plants (belonging to the lines 460 and 16220: Table 1) were tested for *Phoma* resistance. Fungus strains and inoculation method were the same as described by Sacristán and Gerdemann (1986). As expected, all tested plants reacted as resistant.

The selection of the *B. nigra* material was based on the reaction against clubroot *(Plasmodiophora brassicae)*. For this, a collection of 62 lines was tested by inoculation with a *P. brassicae* pathotype which was virulent for all *B. napus* differentials of the European Clubroot Differential Set (ECD code: 19/31/31). The inoculation method was as described by Diederichsen et al. (1989). Six resistant lines (Table 1) were selected and used, after propagation as sterile shoot cultures, for transformation experiments.

Transformation of Brassica nigra

A total of 19 experiments of protoplast co-cultivation with *Agrobacterium tumefaciens* strain C58 pGV 3850 HPT were carried out. Nine experiments were successful with respect to recovery of transformants. They are summarized in Table 2. The hygromycin concentration used for selection (25 μg/ml, 35 μg/ml in Experiments 7 and 8) was lethal for both *B. nigra* seedlings and protoplast colonies (Sacristán et al. 1988). The number of resistant callus-colonies was determined 8–12 weeks after starting selection.

Essential for the recovery of transformed colonies was a high division rate from the beginning of the protoplast culture; it had to be at least about 20% at 4th culture day, otherwise the damage by the bacteria was total. Not all colonies growing at the first selection phase (bead culture, Fig. 1 A) were stably transformed. After individual transfer to agar-hygromycin medium, only a part of them survived and grew to callus. No stably transformed callus could be obtained from lines 229 and 461.

Selected calli grew on hygromycin containing medium as vigorously as control calli on non-selective medium. The majority of them (75%) expressed the nopaline synthase gene (Table 2, Fig. 1 B).

Because the differences found in the values for transformation frequencies (Table 2) could not be attributed to genotypical difference alone – division rate and general condition of the protoplast population after the co-culture played also an important role – no average values, but the results of each single experiment are given (see also Discussion).

So far, only one transformed line from Experiment no. 8 regenerated to plants. Although these derived from a nopaline-positive callus, their leaves did not express the nopaline gene (data not shown).

Table 2. Transformation experiments by co-culture of protoplasts from *B. nigra* with *A. tumefaciens* strain C58 pGV 3850 HPT

Exp. no.	*B. nigra* line	No. plated ppl.	Survival after co-cult. (%)[a]	Hygromycin-resistant calli	Transformation rate[b] (relative) (%)	Nop^+ calli/ total tested
1	460	7.2×10^6	30	27	2.6	20/27
2	460	3.6×10^6	15	1	2.8	1/1
3	460	10^7	67	16	2.2	9/16
4	460	7.8×10^6	20	19	6.8	11/12
5	2051	1.5×10^7	23	9	4.9	6/9
6	2051	1.7×10^7	60	57	2.2	45/51
7	16220	6.6×10^6	60	1	1.0	1/1
8	2058	8.4×10^6	30	10	1.8	1/8
9	2058	10^6	20	4	not. det.	3/4

[a] Percentage of growing colonies from co-cultured cells in non-selective medium with respect to controls (not co-cultivated cells)
[b] Relative transformation rate: % hygromycin resistant calli relative to number of developed calli from co-cultures grown on non-selective medium

Table 3. Results of four fusion experiments between X-ray irradiated protoplasts of Hyg^R-transformed *B. nigra* callus and mesophyll protoplasts of *B. napus*

Exp. no.		No. plated ppl. (of each partner) on selective med.	Plating effic. on non-select. medium	No. colonies growing on selective medium	Nop^+ calli/ total tested	No. regenerating fusion products
1	FT[a]	10^6	>5%	109	54/54	42
	−FT	2.5×10^5		0		
2	FT	1.4×10^6	>5%	92	10/12	6
	−FT	1.3×10^5		0		
3	FT	8×10^5	0.2%	3	not det.	1
	−FT	8×10^4		0		
4	FT	1.7×10^7	>5%	261	16/16	3[b]
	−FT	1.6×10^6		2		

[a] FT – fusion treatment; −FT – no fusion treatment
[b] Regeneration process not concluded

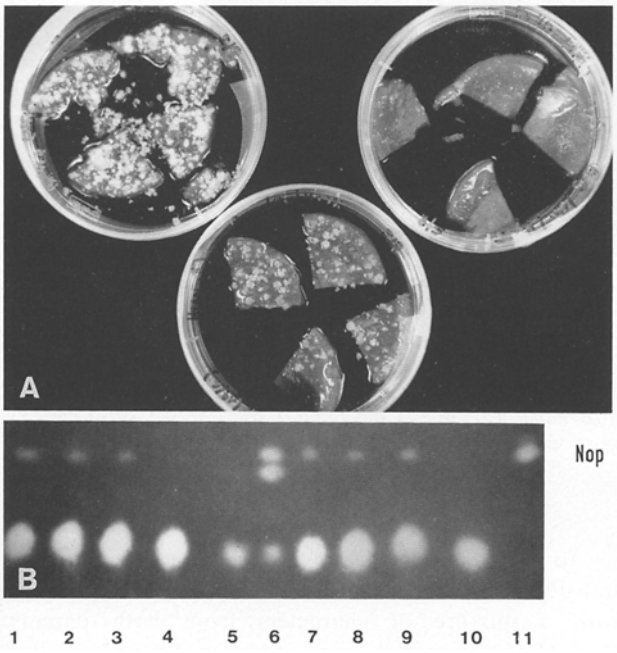

Fig. 1. A Selection of hygromycin resistant colonies, first selection phase; left: control cultures (not co-cultivated) on non-selective medium; right: control cultures on selection medium (25 mg/l hygromycin); bottom: growing colonies on selection medium after co-cultivation with *A. tumefaciens* C58 pGV 3850 HPT. **B**: Nopaline test of eight hygromycin resistant clones. Lane 5: arginine standard; lane 6: nopaline (Nop) + octopine + arginine standards; lane 11: nopaline standard. Lanes 1–4 and 7–9: nopaline positive clones (lane 4, low level of expression); lane 10: nopaline-negative clone

Asymmetric protoplast fusion

In previous tests with protoplasts of *B. nigra* lines 460 and 16220, it could be stated that at irradiation doses of 200 Gy or more, no colonies from the treated protoplasts could be recovered, even though cell wall regeneration took place (data not shown).

In all cases reported here the donor material was a transformed callus line growing rapidly on hygromycin, obtained in transformation Experiment no. 1 (Table 2). Depending on the experiment, between 5% and 20% of the protoplast population – observed immediately after the fusion treatment – was involved in fusion events. The survival after fusion was more than 60%.

Table 3 documents the results of four fusion experiments. Donor protoplasts in Experiments 1–3 were irradiated with 200 Gy. In Experiment 4, two irradiation doses (200 and 300 Gy) were applied.

In Experiments 1–3, no macroscopical colonies from protoplast mixtures which had not been subjected to fusion treatment (controls) developed in selective medium (Fig. 2A). In Experiment 4, two colonies from controls grew in hygromycin medium (200 Gy irradiation of the donor protoplasts); it has to be determined whether they are products of spontaneous fusions or escapes from *B. nigra*. The number of hygromycin-resistant calli recovered from Experiment 4 was higher in the cultures with 200 Gy-irradiated donor protoplasts than in those with 300 Gy irradiation (157 and 104 calli, respectively).

Nearly all selected fusion products analysed for nopaline synthase activity expressed the nopaline gene (Table 3, Fig. 2B).

Confirmation of the hybrid character by isozyme analysis

Because to date no plant could be regenerated from the transformed *B. nigra* callus line used for fusion, parent material and selected fusion products were analysed for the most part as callus. The patterns of esterase activity permit a clear differentiation between *B. nigra* and *B. napus* parent material both as callus and leaves, and thus an identification of fusion products.

Fifty-three different putative fusion products, together with the respective parents, have been analysed for

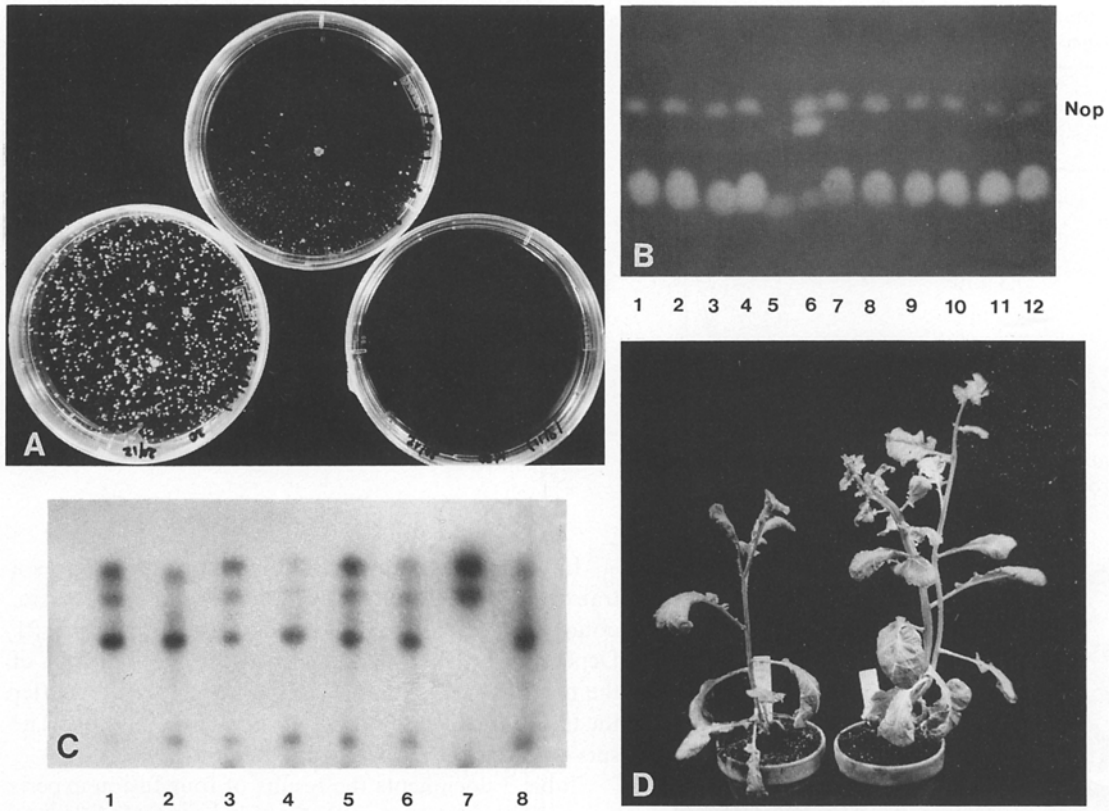

Fig. 2. **A** Selection of fusion products between irradiated protoplasts of hygromycin-resistant *B. nigra* callus and *B. napus* mesophyll protoplasts, 7 weeks after protoplast isolation. Left: colonies from a culture of mixed parent protoplasts not subjected to fusion treatment on non-selective medium (positive control); right: the same on selective medium (negative control); middle: growing colonies on medium with 25 mg/l hygromycin after fusion treatment. **B**: Nopaline test of ten selected fusion products (lanes 1–4 and 7–12), all nopaline positive. Lane 5: arginine standard; lane 6: nopaline + octopine + arginine standards. **C**: Esterase isozyme patterns of six selected fusion products as calli (lanes 1–6) and of the parent material *B. napus* leaf (lane 7) and *B. nigra* transformed callus (lane 8). **D**: Asymmetric somatic hybrids regenerated from two different fusion products

esterases by starch gel electrophoresis. The majority of them clearly contained bands from both parents, which demonstrates their hybrid nature (Fig. 2C). One prominent band from *B. nigra* was present in nearly all the hybrids analysed.

Regeneration of somatic hybrids and chromosome numbers

The redifferentiation of a considerable number of fusion products has been achieved through somatic embryogenesis applying a two-step procedure ('Materials and methods'). This took more time than desired because an immediate transfer of selected colonies to R1 medium led to their degeneration. An established rapid growth on 2N medium was necessary before transferring to embryogenesis-inducing medium. Moreover, the majority of fusion products needed several passages on R2 medium before regenerating shoots could be isolated. In most cases, these shoots were teratoma-like and absent of apical dominance.

To date, 52 plants belonging to 21 selected calli were transferred to soil. They show an abnormal morphology with a mixture of characters from both parents (Fig. 2D). Although there are interclonal morphological differences, all plants have some common features: growth pattern and flower size are rape-like, whereas the abundant pilosity correspond more to the mustard type. Siliqua, for the most part sterile, have an intermediate size, and in one clone their wide shape does not correspond to any parent but resembles that of the related species *B. carinata*.

The chromosome numbers of the parent material are $2n = 16$ for *B. nigra* and $2n = 38$ for *B. napus*. Chromosome counts made in root tips of regenerated plants from selected fusion products at different developmental stages (first before transfer to soil, then in soil) showed a great inter- and intraclonal variation. Single plants were mostly mixoploid, presenting two ploidy levels and/or different chromosome numbers even in a single root (Table 4).

Table 4. Chromosome numbers of the parent material and the first obtained asymmetric somatic hybrids (Exp. 1) planted in soil

Parents	Clone no.	Chromosome no.[a]					
B. nigra (2n)		16					
B. napus (2n)		38					
		~Diploid level				~Tetraploid level	
	1		48				
	2		46	50±1		~75	
	3	36	42±1			78±2	
	4			56	62		
	5		44			74	81±1
	6	35				70	80±1
	7		46	55±2		75	
	8					78±1	81±1
	9		46			78	
	10		44				88±1
	11					75	
	33	38			68	75	

[a] More frequent underlined

Discussion

The stable introduction of the selectable marker hygromycin resistance in several *B. nigra* lines has been achieved by protoplast co-cultivation with *Agrobacterium tumefaciens* C58pGV 3850 HPT. As shown in a previous work (Sacristán et al. 1988), the type of bacterial construct was decisive for the transformation success of this species, a conclusion which is also applicable to transformation attempts with naked DNA in relation with the plasmid used (Köhler et al. 1988).

Although the plant genotype within the species might have played a role in the transformation success or frequency (from two lines of *B. nigra* it was not possible to recover transformants), this dependence is in our case probably indirect, through the condition of the protoplast population. In fact, because of the damage by the bacteria, transformants could be obtained only from protoplast cultures with an early high division frequency. This also explains the different values for transformation rates of the same line from one experiment to other.

More than 75% of the protoplast-calli selected for hygromycin resistance expressed the nopaline synthase gene. Nopaline$^-$ lines grew on hygromycin medium as vigorously as Nop$^+$ lines. It is known that opine genes do not always express or only at very low levels, due to DNA methylation (Hepburn et al. 1983; Peerbolte et al. 1986). This could also be the case for some *B. nigra* clones, which were first Nop$^-$ and became Nop$^+$ after a short culture on medium containing 5-azacytidine.

The incorporation of a selective marker in the *B. nigra* genome was done with the idea of developing a selection system for asymmetric somatic hybrids between this species as donor and *B. napus*, the oilseed rape. Because the *B. nigra* lines used for transformation contain resistance genes against two important rape pathogens *(Phoma lingam, Plasmodiophora brassicae)*, the production of such hybrids should serve to study the possibilities of transferring agronomically relevant genes by asymmetric protoplast fusion.

With the irradiation of the fusion partner *B. nigra*, we aimed to recover asymmetric hybrids with small parts of its genome – containing such genes among them for pathogen resistance – in an unaltered *B. napus* host genome. Müller-Gensert and Schieder (1987) demonstrated that marker genes introduced by *Agrobacterium*-transformation can be transferred by protoplast fusion from a mitotically inactivated donor into recipient protoplasts, and that in this process, besides the T-DNA, other genes of the donor genome are co-transferred.

The results of the present study demonstrate that the selection system used, namely the hygromycin resistance after protoplast fusion, was effective for an unequivocal recovery of somatic hybrids. The hybrid character of the selected calli and regenerated plants has been confirmed by isozyme analysis. Not only the hygromycin resistance marker, for which fusion products were selected, is present in the hybrids, but also the marker nopaline synthase. This indicates that the T-DNA might not have undergone important changes during and after the process of protoplast fusion.

The number of regenerated plants so far obtained does not permit general conclusions to be drawn about all potential hybrids; the regeneration process is to date not yet concluded. But the first regenerants obtained (from 21 fusion products) show some common features. All have morphological characters from both parents (e.g. growth pattern and flower size rape-like, hairiness mustard-like).

The morphology and the isozyme patterns of the hybrids indicate that relatively high amounts of the donor genome have still been retained in the fusion products. This is in accordance with results reported by Imamura et al. (1987) for hybrids between *Nicotiana* and *Hyoscyamus* and by Sidorov et al. (1987), who found that even at irradiation doses which inhibited cell division in *Solanum* true somatic hybrids could be recovered. A stronger irradiation than that required for the mitotic inactivation of *B. nigra* protoplasts reduced the number of hybrid calli produced, as found also by Imamura et al. (1987) for the production of asymmetric hybrids of other species. It has to be determined if the hybrids from fusion products in which the inactivated partner was more heavily irradiated contain lower donor genomic amounts.

True somatic hybrids between *B. nigra* (2n = 16) and *B. napus* (2n = 38) should contain 54 chromosomes if the parent material is euploid. For asymmetric hybrids, it would be expected in the diploid level chromosome numbers lower than 54. This is in accordance with the results obtained relative to the first produced hybrid plants and an indication of their asymmetric hybrid character. In addition, most of the somatic hybrid plants were mixoploid. This reflects a chromosomal instability which can be a consequence of the participation of an irradiated partner in the fusion product, but could also have been originated during the time of growth as callus. Because of the difficulty in distinguishing the chromosomes of the parents from each other, it is not possible to assign a defined genomic contribution of the parent protoplasts in the asymmetric somatic hybrids.

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