Theor Appl Genet (1987) 73:379–384

Somatic hybrids between *Brassica oleracea* and *B. campestris*: selection by the use of iodoacetamide inactivation and regeneration ability

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Received June 24, 1986; Accepted August 30, 1986
Communicated by K. Tsunewaki

Summary. An efficient procedure for obtaining somatic hybrids between *B. oleracea* and *B. campestris* has been developed. Hypocotyl protoplasts of *B. oleracea* were fused with mesophyll protoplasts from three different varieties of *B. campestris* by the polyethylene glycol-dimethylsulfoxide method. The selection of somatic hybrids utilized the inactivation of *B. oleracea* protoplasts by iodoacetamide (IOA) and the low regeneration ability of *B. campestris*. The efficiency of recovery of somatic hybrids depended upon the IOA concentration, and when 15 mM IOA was used, 90% of the regenerated plants were found to be hybrid. The somatic hybrids were examined for i) leaf morphology, ii) leucine aminopeptidase (LAP) isozyme and iii) chromosome number. All the hybrids had intermediate leaf morphology and possessed LAP isozymes of both parental species. The chromosome analysis revealed a considerable variation in chromosome number of somatic hybrids, showing the occurrence of multiple fusion and chromosome loss during the culture. Some of the hybrids flowered and set seeds.

Key words: *Brassica* – Protoplast fusion – Somatic hybrid – Selection

Introduction

Protoplast fusion provides unique opportunities to study cytoplasmic inheritance (Hanson et al. 1985) and has revealed extensive rearrangements of cytoplasmic genomes (Belliard et al. 1979; Medgyesy et al. 1985). In addition, somatic hybridization provides the possibility of transferring genetic factors between sexually incompatible species (Harms 1983).

In the Brassicas, Schenck and Röbbelen (1982) first produced somatic hybrids between *B. oleracea* and *B. campestris* without employing any selection method. More recently, the mechanical isolation of heterocytoplasmic protoplasts was applied and one somatic hybrid between the above two species was obtained (Sundberg and Glimelius 1986). Cybrids of *B. napus* possessing the male sterile trait from one parent and atrazine resistance from the other were obtained, and the transfer of cytoplasmic traits became possible by protoplast fusion in *Brassica* species (Pelletier et al. 1983).

Although a variety of methods have been described for the selection of somatic hybrids (Widholm 1982), none, except for mechanical isolation, has been applied for *Brassica* species up to the present. Therefore, selection methods have to be developed in order to utilize somatic hybridization as a tool for the improvement of these species.

We report here that the combination of iodoacetamide inactivation and regeneration ability provides an efficient selection of somatic hybrids between *B. oleracea* and *B. campestris*. Also, chromosome analysis revealed that a variation in chromosome number among somatic hybrids was due to multiple fusion of protoplasts and chromosome loss during culture.

Materials and methods

Plant materials

B. oleracea var. 'capitata' cv. 'Shutoku' (cabbage) was used as one parent and three different varieties of *B. campestris*, var. 'rapa' cv. 'Tokyo Komatsuna' (komatsuna), var. 'rapa' cv. 'Misugi Komatsuna' (komatsuna), var. 'pekinensis' cv. 'Michihli' (chinese cabbage) and an atrazine-resistant biotype of *B. campestris* (bird's rape), were used as fusion parents. Atrazine-resistant *B. campestris* was provided by Y. Ohkawa (The Institute of Agrobiological Resources, Tsukuba).

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Protoplast isolation

B. oleracea: Hypocotyl protoplasts were isolated according to the method of Glimelius (1984) with minor modifications (Yamashita and Shimamoto 1986). The enzyme solution consisted of 1% Cellulase Onozuka RS (Yakult, Japan), 0.01% Pectolyase Y23 (Seishin Pharmaceutical Co., Tokyo, Japan), and 0.4 M sucrose.

B. campestris: Mesophyll protoplasts were isolated from leaves of aceptically-grown young plants. Surface-sterilized seeds were germinated and grown for 3 weeks on a shoot culture medium containing inorganic salts (NN69: Nitch and Nitch 1969), vitamins (NN67: Nitch and Nitch 1967), 3% sucrose and 0.8% Bactoagar, in a plastic container under light (3,000 lux). After placing the plants in the dark for 3 days, leaves were collected, cut into small pieces and incubated in a pre-enzyme treatment (PET) solution (a 25% concentration of NN67 inorganic salts, vitamins of NN67, 0.35 M sucrose, 0.5 mg/l 2,4-dichloroacetic acid, 0.5 mg/l naphthalene acetic acid, 1 mg/l 6-benzylaminopurine, pH 5.5), overnight at 10 °C. After incubation, the PET solution was replaced with an enzyme mixture (0.5% Cellulase Onozuka R-10 Yakult, Japan, 0.05% Macerozyme Onozuka R-10 Yakult, Japan, and 0.005% Pectolyase Y-23 in the PET solution) and incubated for 2.5–3 h at 30 °C with slow shaking. After removing undigested materials by filtration through a nylon mesh, the enzyme solution was centrifuged (800 rpm, 8 min) and the floating protoplasts were collected and washed twice in the washing solution (50% concentration of NN67 inorganic salts, vitamins of NN67, 0.35 M sucrose, pH 5.5) by centrifugation.

Protoplast fusion, culture and plant regeneration

B. oleracea protoplasts were treated with various concentrations of iodoacetamide (IOA) for 15 min at 4 °C. The stock solution of IOA (50 mM) was prepared in a W5 solution (Menczel and Wolfe 1984) and filter-sterilized. After the IOA treatment, protoplasts were washed with the W5 solution three times by low speed centrifugation. The fusion method was essentially that of Menczel and Wolfe (1984). Four drops (25 or 50 µl per drop) of the fusion solution were placed on the bottom of a plastic dish and an equal amount of mixed protoplast suspension ($3–4 \times 10^6$/ml) was gently placed on the top of the fusion solution. Ten minutes later, 6 or 15 ml of W5 solution containing 5 mM morpholinoethane sulfonic acid (MES), pH 5.6, was slowly added and the dish was kept at room temperature for 2 h. Treated protoplasts were washed once with W5 solution and cultured ($1.5–2 \times 10^5$/ml) in a modified KM8p medium (Kao and Michayluk 1975). Protoplasts were cultured according to Glimelius (1984) with minor modifications. For colony growth, 0.05% Gelrite (Kelco, USA) was used instead of agarose and the medium for shoot induction contained 0.1 mg/l indole acetic acid, 5 mg/l zeatin, 0.25% Gelrite. Shoots were transferred to B-5 medium (Gamborg et al. 1968) containing 0.03 mg/l GA, 0.01 mg/l NAA, 2% sucrose and 2% Agarose type I (Sigma) for further elongation and rooting.

Chromosome analysis

Chromosomes of regenerated plants were analyzed with root tip cells according to the method previously described (Nishibayashi and Kaeriyama 1986).

Starch gel electrophoresis

Leaf extracts of regenerated plants were prepared, electrophoresed and stained for leucine aminopeptidase (LAP) activity according to Arús and Orton (1983).

Results

Effect of dimethylsulfoxide (DMSO) concentration on fusion frequency

In order to obtain a high fusion frequency, the effect of the DMSO concentration on the fusion frequency was examined. As described previously (Haydu et al. 1977; Menczel and Wolfe 1984), the addition of DMSO to the PEG solution markedly increased the frequency of fused cells. The optimal DMSO concentration was found to be 10 or 15% (Table 1). The DMSO concentration higher than 15% tended to damage mesophyll protoplasts. The preparation of stable mesophyll protoplasts by treating leaf slices in the PET solution prior to the enzyme treatment was found to be essential to achieve the high fusion frequency. Fused protoplasts were easily identified because they contained chloroplasts from *B. campestris* and cytoplasmic strands from *B. oleracea* (Fig. 1 A, B).

Selection of somatic hybrids

1 Selection scheme. Hypocotyl protoplasts of *B. oleracea* divide efficiently and regenerate plants from 10–15% of calli placed on the regeneration medium (Yamashita and Shimamoto 1986). Here, their division was inhibited by adding IOA. *B. campestris* protoplasts divide efficiently (ca. 20% division frequency) but are not able to form shoots under the conditions used in this study. Therefore, the colonies formed are either from the hybrid cells or from *B. campestris* protoplasts, and only hybrid calli can regenerate plants (Fig. 1 C, D, E). Some of the hybrids flowered and set seeds (Fig. 1 F).

2 Effect of IOA concentration on recovery of somatic hybrids. The IOA concentration was important in order to obtain hybrids efficiently. As shown in Table 2, with 15 mM IOA, 9 out of 10 regenerated plants were found to be somatic hybrids. However, when the IOA concentration was lower than 10 mM, more than 50% of the

Table 1. Effect of dimethylsulfoxide (DMSO) concentrations on fusion frequency

		DMSO conc. (%)				
		0	5	10	15	20
Fusion frequency[a] (%)	Exp. 1	2.0	6.0	12.1	13.7	10.0
	Exp. 2	0.2	2.1	13.8	13.8	12.7

[a] Fusion frequency was determined 2 h after the fusion treatment. Various concentrations of DMSO was added to 10% PEG solution

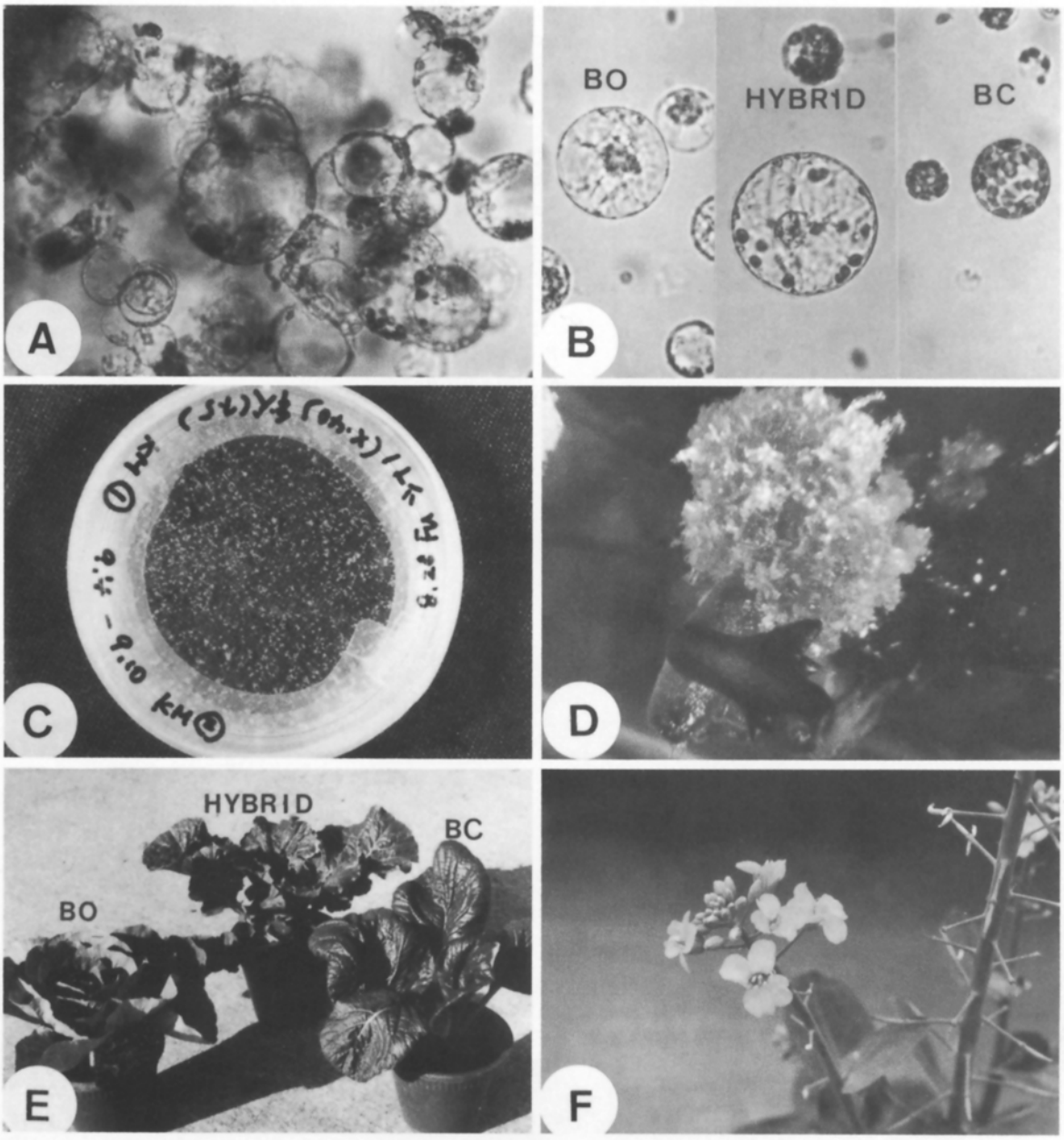

Fig. 1A–F. The fusion, culture and regeneration of somatic hybrids between *B. oleracea* (BO) and *B. campestris* (BC). **A** Fusing protoplasts at 3 min after the start of the fusion treatment; **B** a heterocytoplasmic protoplast 2 h after the fusion treatment; **C** calli from the fusion treated protoplasts; **D** the shoot formation from a hybrid callus; **E** a hybrid plant showing intermediate plant morphology; **F** flowers of a somatic hybrid F3

plants turned out to be *B. oleracea* escapes, although *B. oleracea* protoplasts cultured alone were totally inactivated with 7.5 mM IOA. The treatment of *B. oleracea* protoplasts with even 20 mM IOA had no effect on the fusion frequency (data not shown).

Analysis of somatic hybrids

A total of 22 somatic hybrids were obtained and analyzed as follows.

1 Leaf morphology. A leaf of the somatic hybrid between *B. oleracea* and *B. campestris* var. 'rapa' cv. 'Misugi Komatsuna' is shown in Fig. 2A. All the hybrids showed intermediate leaf morphology, color and texture between *B. oleracea* and *B. campestris.* The hybrids between *B. oleracea* and *B. campestris* var. 'pekinensis' possessed a hairy comb on the leaf, petiole and stem which is characteristic of the *B. campestris* parent. This was also true for the hybrids between *B. oleracea* and another *B. campestris* (bird's rape).

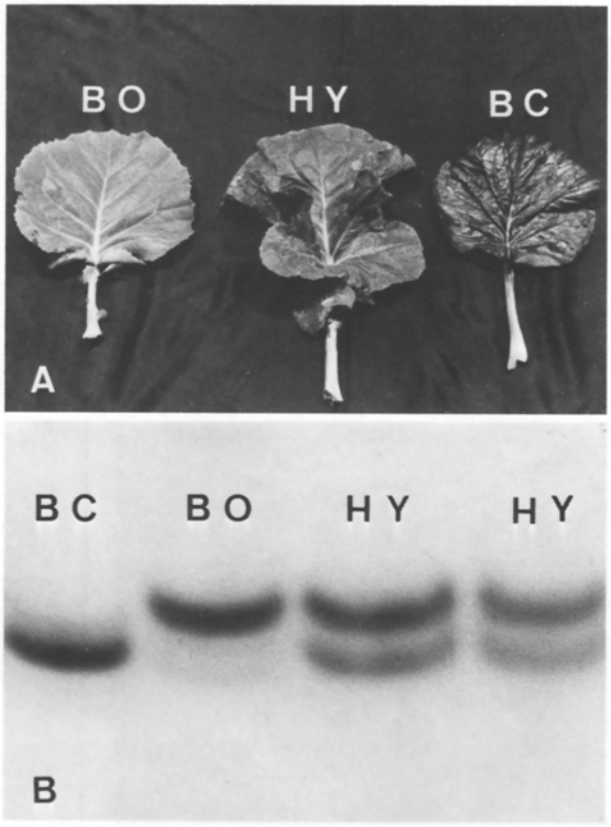

Fig. 2 A, B. Analysis of somatic hybrids. **A** leaf morphology of a somatic hybrid (HY) and the parents, *B. oleracea* (BO) and *B. campestris* var. 'rapa' cv. 'Misugi Komatsuna' (BC); **B** LAP isozyme patterns of somatic hybrids and the parents

2 LAP isozyme. As shown in Fig. 2B, the hybrids contained two bands which corresponded to the single bands found in the parental species.

3 Chromosome analysis. Of the 22 hybrids obtained, 10 plants were analyzed for their chromosome numbers (Table 3). The *B. oleracea* (2n = 18) and *B. campestris* (2n = 20) used in this study possess one and two SAT-chromosomes, respectively (Fig. 3A, B). The satellite of *B. oleracea* is smaller than those of *B. campestris.* The SAT-chromosome was used as the marker to identify the chromosomal constitution of hybrid plants. Although all the plants were identified as hybrids by the LAP analysis and the plant phenotype, a considerable variation in the chromosome number was observed. Only three out of 10 plants examined turned out to be amphidiploids having 38 chromosomes (Fig. 3C). Five plants had 56 chromosomes that were presumably fusion products between one *B. campestris* and two *B. oleracea* protoplasts (Fig. 3D). Aneuploidy was found in two plants. The plant with 57 chromosomes was assumed to have resulted from a duplication of one chromosome in the 56 chromosome plant. The hybrid

Table 2. Effect of iodeacetamide (IOA) concentrations on recovery of hybrid plants[a]

	IOA conc. (mM)[b]					
	0	2.5	5	7.5	10	15
Total no. plants	1	17	2	14	7	10
No. hybrids	0	1	1	6	5	9
No. *B. oleracea* plants	1	16	1	8	2	1
No. *B. campestris* plants	0	0	0	0	0	0
Hybrid (%)	0	6	50	43	71	90

[a] Results from a series of fusion experiments between *B. oleracea* (cv. 'Shutoku') and three different varieties of *B. campestris* are summarized
[b] IOA treatment was performed for 15 min at 4 °C

Table 3. Chromosome number of somatic hybrids[a]

B. oleracea	*B. campestris*	Plant no.	Chromosome no.
cv. 'Shutoku'	var. 'rapa' cv. 'Tokyo Komatsuna'	F 1	56
		F 2	56
		F 3	57
		No. 1	56
cv. 'Shutoku'	var. 'rapa' cv. 'Misugi Komatsuna'	416-4	56, 36
		416-5	49, 33
		516-1	38
		516-2	38
cv. 'Shutoka'	var. 'pekinensis' cv. 'Michihli'	412-11	38
cv. 'Shutoku'	Bird's rape	725-3	56

[a] Hybrid nature of all the plants was identified by the LAP analysis and the plant morphology

416-5 had an unexpected chromosome number that suggested, together with the size of satellites, its origin from the hybrid with 56 chromosomes by extensive chromosome loss. The plants 416-4 and 416-5 were chimeric in chromosome number and two distinct cell types with different chromosome numbers were found. Three plants, F3, No. 1 and 416-4, flowered by this time, among which plant F3 set normal seeds.

Discussion

The PEG-DMSO method of protoplast fusion employed in this study is far superior to other PEG methods in fusion frequency and the viability of treated protoplasts. To obtain the reproducible high fusion frequency, "strong" mesophyll protoplasts of *B. campestris* with low buoyant density have to be isolated. Such protoplasts are able to contact with light hypocotyl

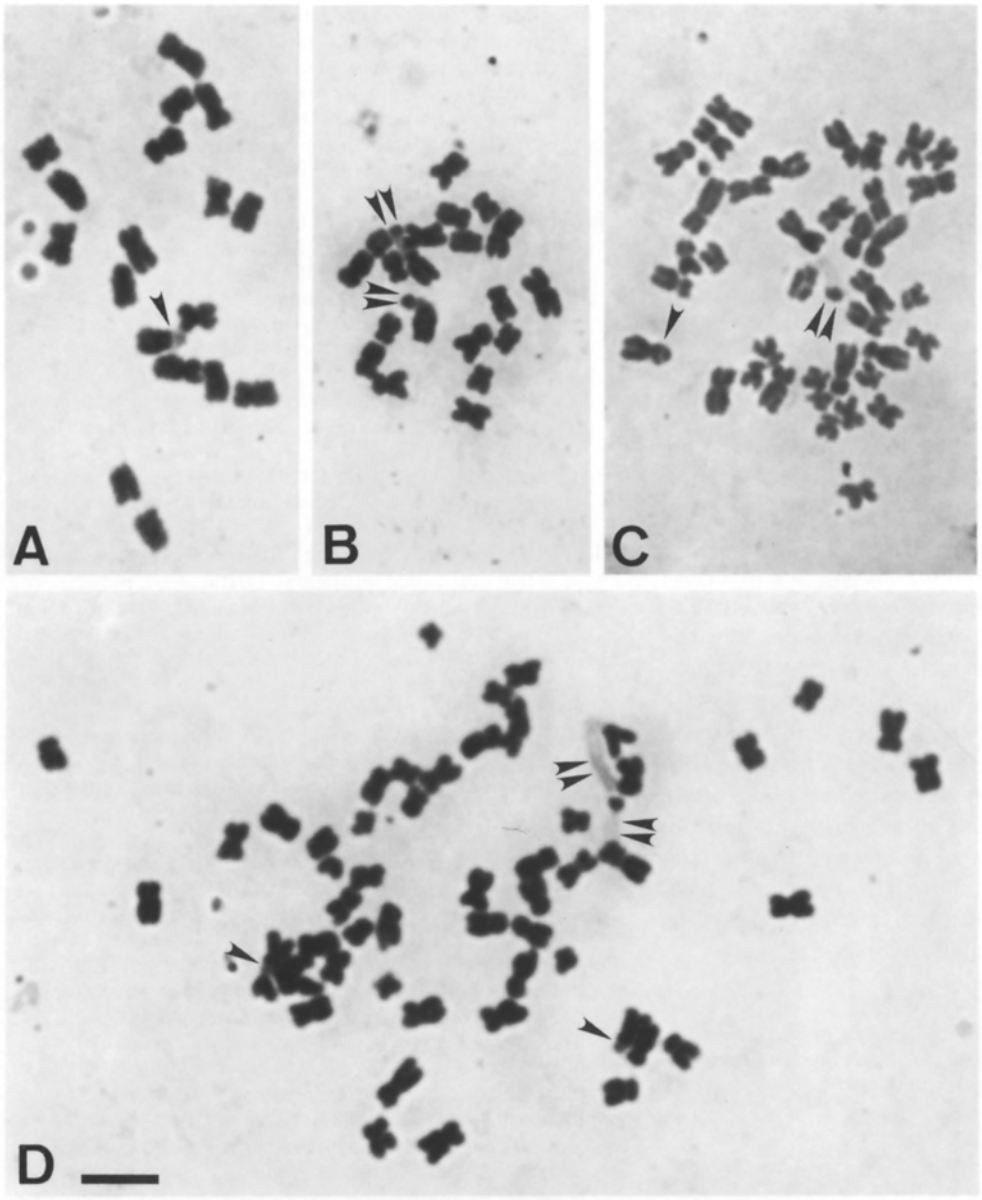

Fig. 3A–D. Chromosome analysis of somatic hybrids. **A** *B. oleracea* (2n = 18); **B** *B. campestris* var. 'rapa' cv. 'Tokyo Komatsuna' (2n = 20); **C** a somatic hybrid 412-11 with 38 chromosomes; **D** a somatic hybrid, F2, with 56 chromosomes. *Arrowheads* indicate satellites (*B. oleracea, single arrowhead; B. campestris, double arrowheads*). Bar = 5 μm

protoplasts of *B. oleracea* during the fusion treatment. This was achieved by the pretreatment of *B. campestris* leaves with the PET solution before the enzyme digestion.

When the parental species have no selective markers at the cellular level, efficient procedures for the hybrid cell selection must be devised. In this study, IOA treatment (Nehls 1978) of the protoplasts of one parental species (*B. oleracea*) was successfully combined with the low regeneration ability of the other parent (*B. campestris*). The IOA concentration was most important for the efficient recovery of somatic hybrids. The IOA treated *B. oleracea* protoplasts occasionally divided when they were cocultured with non-treated *B. campestris* protoplasts, even at the IOA concentration high enough to kill all the *B. oleracea* protoplasts when cultured alone. This "nurse effect" of non-treated protoplasts explains the appearance of a *B. oleracea* escape even at the 15 mM concentration of IOA (Table 2).

The IOA treatment can be applied in combination with other inactivating agents such as diethylpyrocarbonate (Nehls 1978) and X-ray (Sidorov et al. 1981) to further simplify the hybrid selection. Experiments to obtain asymmetric fusion

products in *Brassica* species by using such a double inactivation are in progress.

A low regeneration ability of one parental species has been utilized for selection of somatic hybrids (Kameya et al. 1981). Plant regeneration from *B. campestris* protoplasts is very rare (Glimelius 1984). There are no reports on plant regeneration from vars. 'rapa' and 'pekinensis' protoplasts used in this study. On the shoot induction medium, two types of calli, i.e., the fast-growing soft type and the slow growing compact type, were formed. Only the former type calli produced shoots, the latter (presumably *B. campestris* calli) never forming them. The hybrid nature of regenerated plants is easily identified by a simple LAP analysis with a small leaf sample (ca. 10 mg). Callus materials could be also used which should enable the prescreening of hybrid calli.

The chromosome analysis of somatic hybrids revealed a considerable variation among the hybrids. Eight out of 10 plants examined were expected euploids. The plants with 56 chromosomes possibly resulted from multiple fusion (2 *B. oleracea* + 1 *B. campestris*) of protoplasts. The chromosome loss and addition found in somatic hybrids indicated that the karyotypes of some somatic hybrids were unstable. This result was in contrast with our previous observation that there were no aneuploids among 21 plants derived from mesophyll protoplasts of *B. oleracea* (Yamashita and Shimamoto 1986, unpublished). Further analysis of somatic hybrids should reveal the extent of karyotypic changes occurring in the somatic hybrids.

Acknowledgements. We thank Y. Ohkawa for the seeds of bird's rape and T. Kameya for the advice on the selection scheme. We also thank colleagues at the Plantech Research Institute for helpful discussions.

References

Arús P, Orton TJ (1983) Inheritance and linkage relationships of isozyme loci in *Brassica oleracea*. J Hered 74:405–412

Belliard G, Vedel F, Pelletier G (1979) Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. Nature 281:401–403

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. Exp Cell Res 50:151–158

Glimelius K (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol Plant 61:38–44

Hanson MR, Rothenberg M, Boeshore ML, Nivison HT (1985) Organelle segregation and recombination following protoplast fusion: analysis of sterile cytoplasms. In: Zaitlin M et al. (eds) Biotechnology in plant science. Academic Press, London New York, pp 129–144

Harms CT (1983) Somatic incompatibility in the development of higher plant somatic hybrids. Quart Rev Biol 58: 325–353

Haydu Z, Lazar G, Dudits D (1977) Increased frequency of polyethylene glycol induced protoplast fusion by dimethylsulfoxide. Plant Sci Lett 10:357–360

Kameya T, Horn ME, Widholm JM (1981) Hybrid shoot formation from fused *Daucus carota* and *D. capillifolius* protoplasts. Z Pflanzenphysiol 104:459–466

Kao KN, Michayluk MK (1975) Nutritional requirements for growth of *Vinca hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126: 105–110

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6960–6964

Menczel L, Wolfe K (1984) High frequency of fusion induced in freely suspended protoplast mixtures by polyethylene glycol and dimethylsulfoxide at high pH. Plant Cell Rep 3:196–198

Nehls R (1978) The use of metabolic inhibitors for the selection of fusion products in higher plant protoplasts. Mol Gen Genet 166:117–118

Nishibayashi S, Kaeriyama J (1986) Structural stability of chromosomes in rice (*Oryza sativa* L.) plants regenerated from somatic tissue culture. Plant Tissue Culture Lett 3:31–34

Nitch C, Nitch JP (1967) The induction of flowering in vitro in stem segment of *Plambago indica* L. 1. The production of vegetative buds. Planta 72:355–370

Nitch C, Nitch JP (1969) Haploid plants from pollen grains. Science 163:85–87

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rousselle, Renard M (1983) Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol Gen Genet 191: 244–250

Schenck HR, Röbbelen G (1982) Somatic hybrids by fusion of protoplasts from *Brassica oleracea* and *B. campestris*. Z Pflanzenzücht 89:278–288

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate treated protoplasts. Planta 152:341–345

Sundberg E, Glimelius K (1986) A method for production of interspecific hybrids within *Brassiceae* via somatic hybridization, using resynthesis of *Brassica napus* as a model. Plant Sci 43:155–162

Widholm JM (1982) Selection of protoplast fusion hybrids. In: Fujiwara A (ed) Plant tissue culture. Maruzen, Tokyo 1982, pp 609–612

Yamashita Y, Shimamoto K (1986) Plant regeneration from cabbage protoplasts. In: Bajaj VPS (ed) Biotechnology of crop improvement. Springer, Berlin Heidelberg New York Tokyo (in press)