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Somatic hybrids between *Brassica juncea* (L). Czern. and *Diplotaxis harra* (Forsk.) Boiss and the generation of backcross progenies

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Abstract An attempt to transfer genes from drought-tolerant *Diplotaxis harra*, a wild relative of *Brassica* species, to an elite oil-yielding cultivar, B-85, of mustard (*Brassica juncea*) was made through protoplast fusion, as the two plant systems are sexually incompatible. By following the standard protocol for PEG-mediated protoplast fusion followed by high pH, high Ca^{++}, DMSO treatment and appropriate cell-culture technique, 16 presumptive somatic hybrid plants could be regenerated. Chromosomal analysis of four such somatic hybrids revealed that three of them were asymmetric. Analysis of morphological characters, meiotic chromosomes, and esterase isoenzyme pattern revealed that all the somatic hybrids were different from each other. Furthermore four chromosomes of each genome could undergo homoeologous pairing at meiosis indicating the possibilities for genetic recombination and chromosomal rearrangements. Irregular distribution of chromosomes at anaphase-II at meiosis has been a consistent feature of these plants. Eventually, pollen of all the somatic hybrids showed complete infertility preventing the recovery of any selfed seed. Nevertheless, ovule fertility of one somatic hybrid was not totally impaired as it had set some seeds upon backcrossing with the *B. juncea* parent. The esterase isoenzyme banding pattern of 24 individual progeny plants of this backcross provided evidence for their recombinant nature. It was thus confirmed that a transfer of genetic traits from *Diplotaxis harra* to *B. juncea* had indeed taken place. Furthermore, it was conceptualised that a transfer of alien genes through the protoplast-fusion technique is primarily possible in situations where meiotic pairing of the chromosomes of the two participating genomes generates recombinant gametocytes which can pass through subsequent filial generations.

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Introduction

Somatic hybrid plants are hybrids derived from the fusion of somatic cells. Regardless of genetic relatedness, it is possible to achieve fusion between protoplasts of different plant systems. Theoretically such fusions offer unlimited possibilities for genetic exchange. The excitement and expectations generated in the 1970s by the potentiality of the protoplast-fusion technique, culminating in the generation of "pomatoes" (Melchers et al. 1978), could not be sustained with any economic utility until the use of the technology in a number of plants belonging to the Brassicaceae (Glimelius et al. 1991). Several somatic hybrids, encompassing the genera *Eruca*, *Sinapis* and *Diplotaxis* with *Brassica juncea* or *Brassica napus* and *Brassica oleracea*, have now been developed. Some fertility has been found in the ovules in such hybrids. Backcrossing with the cultivated parent type has thus opened up the distinct possibility of recovering genes from the wild species. Infact, through these cases, the success of protoplast fusion could conveniently be translated into a conceptual extension of sexual crosses between plant species which have been separated from each other by a sexual incompatibility barrier. Such fusions have thus extended the scope of plant breeding both at organismic and at cellular levels.

Desirable genetic attributes contained amongst the members of wild crucifers in the genus *Diplotaxis* have attracted attention for quite some time. Attempts have been made in the past to transfer such traits to cultivated species of *Brassica* through protoplast fusion since sexual crossing barriers exist between the two genera. *Diplotaxis harra* (Forsk.) Boiss (2n = 26) is known to be a remarkably drought-tolerant plant system (Mizushima and Tsunoda 1976). Additionally, *Diplotaxis* cytoplasm is also known to contain a cytoplas-

mic male sterility principle (Hinata and Konno 1979). Previous successful protoplast fusion studies have been carried out between *B. napus* + *D. harra* (Klimaszewska and Keller 1988) and *B. juncea* + *D. muralis* (Chatterjee et al. 1988). However, the ultimate objective of introgressing a desirable genetic trait into a *Brassica* cultivar was not realised because the hybrids turned out to be sterile. The present communication deals with the results of our success in generating somatic hybrids between *B. juncea* + *D. harra* and also in being able to generate progenies when backcrossed with the cultivated *B. juncea* parent.

Materials and methods

Protoplast isolation

Seeds of *B. juncea* (L.) Czern cv B-85 were surface sterilized with 0.2% $HgCl_2$ for 5 min followed by thorough washing with sterile distilled water. Seeds were cultured in hormone-free MS medium in the dark at 28 °C for 2 days followed by exposure to a 16/8-h photoperiod (light 3000 lx) at 26 °C. The stems from 21–28 day old aseptically grown plants were cut into 0.5–0.8 mm segments. Each segment was halved longitudinally and placed in SC 1 medium [MS basal (Murashige and Skoog 1962) + 0.25 mg/l 2, 4-D + 0.25 mg/l NAA + 0.25 mg/l BAP] and incubated in the same culture conditions. Seven to ten-day-old freshly induced stem callus was used as the source material for protoplast isolation. The stem calli were chopped into pieces in an enzyme mixture containing 1% Pectinase (Serva), 2% Cellulase (Onozuka R-10), 0.6 M mannitol, 0.2% $CaCl_2 \cdot 2H_2O$, pH 5.8. Finally 20 ml of enzyme mixture per gram of fresh weight of callus tissue was used and incubated at 26 °C for 17 h on a horizontal shaker (30 strokes per min). The protoplasts were purified by centrifugation (at 100 g) with washing solution (0.6 M mannitol + 0.2% $CaCl_2 \cdot 2H_2O$).

Seeds of *D. harra* (Forsk.) Boiss (2n = 26) (provided by Prof. K. Hinata, Tohoku University, Japan) were surface sterilized and cultured in the same way as in the case of *B. juncea*. Dark-green leaves from 21–28-day-old plants were used as the source material for protoplast isolation. Seven microliters of enzyme mixture per gram of fresh weight of leaf tissue were used and incubated at 26 °C for 16 h. The composition of the enzyme mixture, the shaking conditions, the protoplast purification and washing were the same as used for *B. juncea*.

Protoplast fusion, culture and plant regeneration

Protoplast fusion was carried out between freshly induced stem callus protoplasts of *B. juncea* and mesophyll protoplasts of *D. harra*. In each case, protoplasts were suspended in washing solution at 1×10^5 protoplasts/ml density. *B. juncea* and *D. harra* protoplasts were mixed in 1:3 ratio. Protoplast fusion was carried out with 30% polyethylene glycol [PEG, MW 4000 (Sigma)] followed by elution with a high pH, 10.5, high Ca^{++} and DMSO solution following the drop method (Chatterjee et al. 1988). After fusion, protoplasts were cultured in 2 ml of liquid MK_3S1 medium [K_3 basal (Kao and Michayluk 1974) medium modified (Chatterjee et al. 1988) + 0.2 mg/l 2, 4-D + 0.2 mg/l NAA + 0.25 mg/l BAP + 0.1 mg/l GA_3 + 0.6 M glucose, pH 5.8] in Petri plates (35 mm diameter). The plates were sealed with parafilm and incubated at 26 °C in the dark for 7 days. After 7 days, the old medium was replaced by fresh medium and exposed to a 16/8-h photoperiod (light 3000 lx) at 26 °C. On the 14th day the old medium was replaced by liquid MK_3S2 medium (MK_3 basal + 0.1 mg/l 2, 4-D + 0.1 mg/l NAA + 1.0 mg/l BAP + 0.1 mg/l GA_3 + 0.4 M glucose + 2% sucrose, pH 5.8). On the 21st day the liquid medium was further replaced by solid medium MSS3 (MS basal + 0.1 mg/l 2, 4-D + 0.1 mg/l NAA + 2.0 mg/l BAP + 0.1 mg/l GA_3 + 3% sucrose + 0.6% Noble agar, pH 5.8). On the 28th day the calli were transferred to R1 plant regeneration medium (0.5 concentration of MS basal medium + 1.0 mg/l NAA + 1.0 mg/l BAP + 0.1 mg/l GA_3 + 0.8% agar, pH 5.8). Following 7 days in regeneration medium, shoot initiation was started in MSR 2 medium (0.5 concentrations of MS basal + 0.5 mg/l NAA + 2.0 mg/l BAP + 0.1 mg/l GA_3 + 0.8% agar, pH 5.8). The shoots were transferred to hormone-free MS medium for rooting and further growth. The clonal replica of each plant regenerated in culture was transferred to the glass house after hardening.

Chromosome analysis

Flower buds of regenerated plants of interest were fixed in 1:3 aceto-ethanol and stored in 70% ethanol. Chromosome analysis of the pollen mother cells of the fixed flower buds were carried out with 2% aceto-carmine staining solution.

Isoenzyme analysis for esterase

The somatic hybrids were analysed for their nuclear-coded esterase isoenzyme pattern. Cell-free extracts were prepared from the leaves of the plant. Polyacrylamide-gel electrophoresis (PAGE) was performed under non-denaturing conditions according to the method of Davis (1964) using a 10% separating gel overlayed with a 3% stacking gel. The slab-gel system of Studier (1973) was used and gels of 18 × 16 × 0.2 cm size were prepared. The running time was 9 h at a constant voltage of 80 V at 4 °C. Isoenzyme banding patterns were detected on the gels after staining for esterases (Brewbaker et al. 1968).

Results

Our protocol resulted in about 75% fusion. The two parental types of protoplasts could be distinguished in many cases because 95% of cv B-85 calli protoplasts were free of chlorophyll, whereas all of the *D. harra* mesophyll protoplasts contained chloroplasts. The protoplast culture and plant regeneration route of Chatterjee et al. (1988) was followed for *B. juncea* cv B-85 in order to recover plants from the fused product (Table 1). This eliminated the possibility of recovering plants of *D. harra*. Products originating from intergeneric fusion and from protoplasts of *B. juncea* were both expected to give rise to plants. A total of 596 calli were exposed to the regeneration medium. From such calli, 250 plants were generated.

Based on morphological features, we were initially able to select 16 plants as presumptive somatic hybrids. These plants were stunted in growth having small, round, stiff leaves with densely arranged large trichomes. Trichomes were also present on the stem. From each node, small branches developed at the early developmental stages. Another distinguishing morphological feature of *D. harra* was the pubescent nature of the surface of the stem, leaf (Fig. 1g) and sepal. Hybrid plants showed an intermediate degree of pubescence. The pollen was sterile in all the plants, though some showed irregular development of the gynoecium (Fig. 1h). Additionally, *D. harra* plants in culture secrete phenolic substances characterized by a dark-brown colouration of the medium. Four plants amongst the

Table 1 Flow chart for basal medium, osmoticum, hormones and other cultural conditions needed for protoplast fusion-derived plant regeneration of *B. juncea* cv B-85 and *D. harra*. In all the cases the pH was maintained at 5.8

Days on which media change done	Media code no.	Basal medium	Molarity of glucose (M)	Percentage of sucrose (%) conc.	Nature of media liquid/solid	Hormones mg/l				Light intensity (lux)	Temp.	Response
						2,4-D	NAA	BAP	GA_3			
0	DH2	MK_3	0.6	–	Liquid	0.2	0.2	0.25	0.1	2500	28 ± 1°C	Cell-wall regeneration 1st and 2nd division
8	DH2	MK_3	0.6	–	Liquid	0.2	0.2	0.25	0.1	2500	28 ± 1°C	Formation of 16–64 cells
15	S2	MK_3	0.4	2	Liquid	0.1	0.1	1.0	0.1	3000	26 ± 1°C	Formation of microcolony
22	S3	MS	–	3	Solid (0.6% agar)	0.1	0.1	2.0	0.1	3000	26 ± 1°C	Formation of macrocolony
28	R1	Half MS	–	1.5	Solid (0.8% agar)	–	1.0	1.0	0.1	3000	26 ± 1°C	Shoot-bud formation
35	R2	Half MS	–	1.5	Solid (0.8% agar)	–	0.5	2.0	0.1	3000	26 ± 1°C	Shoot-bud formation
42	–	MS-H	–	3	Solid (0.8% agar)	Hormone free				3000	26 ± 1°C	Complete plant regeneration

presumptive somatic hybrids showed a characteristic dark-brown colouration at their base. These four plants (SH-4, SH-10, SH-14 and SH-16 as in Fig. 1 b, d, e, f) were thus analysed further; their salient morphological features are documented in Table 2.

The chromosome-number analysis of pollen mother cells of the somatic hybrids indicated that they contained $2n = 58$ for SH-4; $2n = 62$ for SH-10; $2n = 60$ for SH-14 and SH-16. Since the summation of 2n numbers of both of the fusing plant species is 62 asymmetric hybrids were evidently present amongst the somatic hybrids generated. An analysis of diakinesis and metaphase-I revealed from 1 to 4 quadrivalents while in one case a hexavalent was also present. This indicated that homoeologous pairing between different chromosomes must have taken place. This, in turn, suggested that chromosomal rearrangements through crossing over of the meiotic products remained a distinct possibility. Additionally, at anaphase-I and metaphase-II, an irregular distribution of chromosomes was observed.

Esterase isoenzyme analysis (Fig. 2a) showed characteristic banding patterns for the four somatic hybrids; none of them were alike in their band pattern. The esterase band patterns of the two parents (B-85 and *D. harra*) are shown in Fig. 2b. Bands B1, B2 and B11 are considered as characteristic marker bands for B-85. On the other hand, bands D3, D4, D5, D6, D10 and D12 represent the characteristic bands of *D. harra*. B1 was present in SH-4, SH-10 and SH-14 with high activity, but only faintly in the case of SH-16. Band B2 was only present in SH-14 at a low activity level. The B11 band of B-85 was not present in any of the somatic hybrids. On the other hand, bands D3, D4, and D6 were present in SH-4 and also in SH-16, although at a low level; additionally, D12 was present in SH-4. Band D10 was present in SH-10, SH-14 and SH-16, and D7 was present only in SH-4 at a high level. The esterase isoenzyme band pattern of the somatic hybrids showed differences when proteins of B-85 and *D. harra* were artificially mixed (1 : 1). Thus, it was evident that the genes responsible for isoenzyme esterase production in the two genomes interact to produce new bands which were not represented in either of the two parents.

All the somatic hybrids showed complete pollen sterility. When they were backcrossed with both of the parents, only one plant (SH-4), backcrossed with cv B-85, was able to set some seeds indicating its limited ovule fertility. A majority of the seeds, however, were shrivelled and devoid of endosperm growth. Nevertheless, a few seeds could be recovered, which were round, yellowish-brown in colour, and smaller in size compared to B-85 seeds. About 75% of the F_1 seeds germinated and yielded plants. The morphological characters of these plants showed differences amongst each other and also from their parents. Most of the plants were pubescent, a character acquired from *D. harra*. All the plants contained dense trichomes on stem and leaf. The lamina of the leaves were broad and dark green. The margin and shape of the leaves were, however, similar to cv B-85.


a
b
c
d
e
f
g
h

Fig. 1 **a** Protoplast-derived regenerated plants of *D. harra* at flowering in the outdoor condition. **b** Somatic hybrid plant (SH-16). **c** Protoplast-derived regenerated plant of *B. juncea* in the field. **d** A somatic hybrid plant (SH-10). **e** A somatic hybrid plant (SH-4). **f** A somatic hybrid plant (SH-14). **g** Leaf characteristics of B-85 (left) and *D. harra* (right) with somatic hybrids SH-10, SH-16, SH-4 and SH-14. **h** Abnormal development of the gynoecium in SH-14

The esterase isoenzyme pattern of 24 backcross progenies were further analysed. The parental somatic hybrid, SH-4, showed characteristic cv B-85 and *D. harra* bands. Interestingly, the 24 backcross progenies were characterised by recombinant esterase isoenzyme patterns. Each progeny carried certain distinct marker bands of either or both of the parents and many new band patterns. This indicates that the plants carried recombined genetic traits resulting from the introgression of *D. harra* characters into the cv B-85 genome.

Fig. 3a, b Esterase isoenzyme pattern of backcross progenies (1–24) along with the parental somatic hybrid (*SH* somatic hybrid SH-4) and parents (*Dh D. harra, Bj B. juncea*)

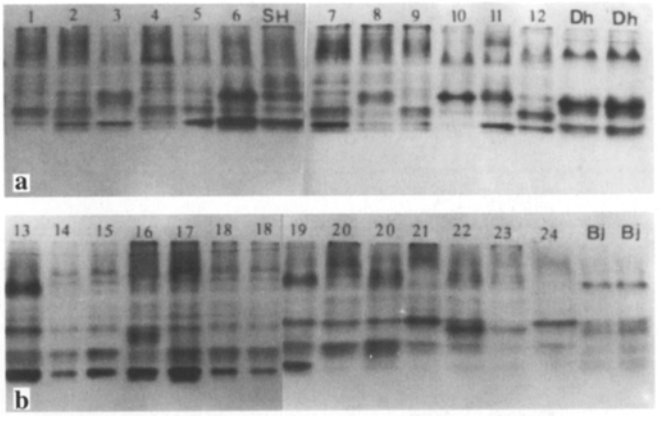

Fig. 2 **a** Esterase isoenzyme pattern. *Lane 1 B. juncea* cv B-85, *2 D. harra, 3* mixture of both B-85 and *D. harra, 4* Somatic hybrid (SH-4), *5* Somatic hybrid (SH-10), *6* Somatic hybrid (SH-14), 7 Somatic hybrid (SH-16). **b** Zymograms of esterase isoenzyme pattern of four somatic hybrids between *B. juncea* cv B-85 and *D. harra*

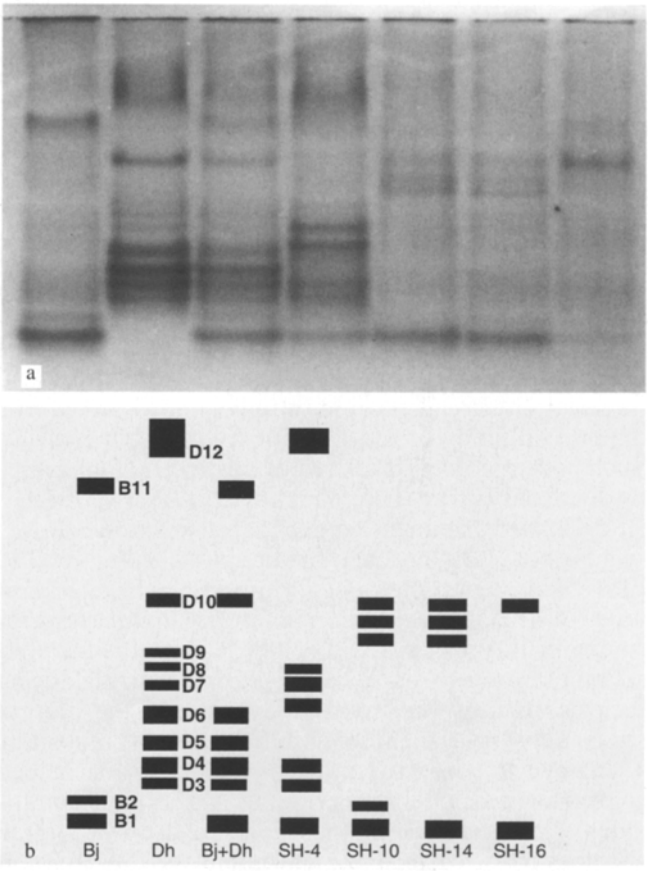

Table 2 Morphological features of somatic hybrids and their parents

Morphological features[a]		SH-4	SH-10	SH-14	SH-16	*D. harra*	*B. juncea* cv B-85
1.	Height of plant	45	90	78	38.5	56.3	50.3
2.	No. of branches	3–5	3–4	4–5	14–17	1–2	2–4
3.	Diameter at the base of the stem	2.1	2.0	1.8	2.1	1.9	2.0
4.	Length of leaf	9.5	12.3	4.5	7.5	9.2	9.5
5.	Length of lamina	7.2	9.4	3.5	5.0	7.5	10.5
6.	Width of the leaf	4.1	5.5	3.4	3.2	3.1	4.2
7.	Internode distance	3–4	5–6	2–3	1–3	1–2	4–5
8.	No. of inflorescences	3–4	3–4	10–12	2–3	3–4	2–3
9.	No. of flowers per inflorescence	20–29	30–42	40–45	30–38	30–72	45–48
10.	Flower morphology	Yellow, like B-85	Yellow, like *D. harra*	Yellow, small abnormal	Yellow, small	Whitish yellow, large	Yellow
11.	Pollen fertility	–	–	–	–	87.5%	89.2%
12.	No. of flowers per plant	76	261	906	58	105	206
13.	Length of the stigma	1.1	1.8	1.5	1–15	1.9–2	3–5
15.	Initiation of flowering in days	65	35	32	81	73	35

[a] Measurements of diameter, height, length and distance are given in cms

Discussion

We have used the protoplast fusion technique to generate somatic hybrids in order to introgress alien genes of *D. harra* into cv B-85, an elite cultivar of *B. juncea*. The amenability to the techniques of protoplast culture and manipulation in plants belonging to the Brassicaceae has in the past resulted in the synthesis of a number of interspecific and intergeneric somatic hybrids. However, many of these hybrids turned out to be sterile, thereby blocking the possibility for gene transfer through a sexual route.

Widely differing plant species when brought together in a heterokaryon are likely to show disharmony at many functional steps during development. This in turn would result in problems for plant regeneration involving irregular development of the floral organs, coupled with loss of fertility and seed formation. There may also be variability in chromosome number and loss of genetic material ultimately resulting in asymmetric hybrids. Such asymmetric hybrids are of particular importance as discussed earlier (Glimelius et al. 1991). The present study, as well as our earlier experience with the *Erussica* (Sikdar et al. 1990) progenies (data not shown), tend to prove this point. Often the asymmetric hybrids contribute towards the restoration of fertility. In fact restoration of fertility may have a direct bearing on the chromosomal rearrangement and elimination occurring during meiosis amongst the somatic hybrids. In the present study SH-4, which had eliminated four chromosomes, was shown to possess ovule fertility which in turn helped to recover seeds after backcrossing. Additionally, multivalent formation during meiotic prophase-I generated the possibility for recombination and the formation of rearranged chromosomes. These rearranged chromosomes could be recovered in a homozygous state to generate novel genotypes. In this scheme, the scope for introgression of novel genes from alien plant systems is limited only to those that are contained in the linkage groups which are capable of undergoing homoeologous pairing and subsequent crossing over. This is the most likely mechanism that can be exploited in the use of protoplast-fusion studies to introgress alien genes from distantly related plant species. The scope for alien chromosome addition or substitution is likely to be extremely limited in this situation as repeated backcrossing will tend to bring the genomic content back to the disomic level. Thus, the utility of the protoplast-fusion technique from the geneticist's point of view should be considered primarily as a way to transfer only a limited quantum of alien genes into the background of the recipient's genotypic make up provided that homoeologous pairing does take place. Such recombinants are likely to form novel genotypes in the gene pool of the crop species, hitherto not available in the sexual world of the crop species.

The present study has demonstrated that the creation of genetic variability in *B. juncea* by introgressing alien genes of *D. harra* through protoplast fusion is a feasible proposition. Indeed the genetic characters of the backcrossed progenies in the present case have shown variability. The profile of esterase isoenzyme pattern revealed extensive variations between plants. This has indicated that the intended introgression of genetic traits of *D. harra* into the genetic background of *B. juncea* cv B-85 has in fact taken place.

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