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Analysis of plants regenerated from protoplast fusions between *Brassica napus* and *Eruca sativa*

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Summary. Protoplasts from etiolated hypocotyls of *Brassica napus* stained with carboxyfluorescein were fused with mesophyll protoplasts from *Eruca sativa*. Hybrid cells could be identified under the light microscope by (1) fully developed chloroplasts derived from *E. sativa* and (2) the cytoplasmic strands of the *B. napus* hypocotyl protoplasts, or (3) by the presence of both red and green fluorescence when investigated under UV light. The heterokaryons were selected using either a micro-manipulator or a flow sorter. On average, 5.4% of the calli obtained after selection differentiated into shoots. Regenerated shoots were subjected to isozyme analysis for verification of their hybrid character. Of the 23 hybrids successfully transferred to the greenhouse, 11 were asymmetric according to isozyme analysis. The nuclear DNA content of the hybrids was determined by flow cytometry, which gives an estimate of chromosome number. Most of the hybrids had a DNA content, and thus a chromosome number, that deviated from the expected sum of the parents. Almost all of the hybrids had some degree of fertility and produced seeds. Seed set, expressed as seeds per pollinated flower, was on average 7% of that of *B. napus* in the case of self-pollination and 26% of that of *B. napus* when backcrossed to *B. napus*. The chloroplast genotype was investigated in 13 hybrids. Of these, 11 had chloroplasts derived from *B. napus*, while only 2 had chloroplasts of *E. sativa origin*.

Key words: *Brassicaceae* – Protoplast fusion – Isozyme analysis – Asymmetric hybrids – Chloroplast DNA

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Introduction

Wild species are often a valuable source of genes important in crop improvement. Various types of sexual barriers can, however, severely restrict the utilization of germplasm from species distantly related to the crop plant. An alternative to the sexual production of hybrids is somatic hybridization. In this technique, the incompatibility barriers between species can be overcome, and a widening of the gene pool of a domesticated crop can be obtained.

In the *Brassicaceae* family, somatic hybridization could be the way for achieving crop improvement, since sexual barriers in many cases (Downey et al. 1980) have made it difficult to effectively transfer traits of interest through sexual hybridization. Several somatic hybrids have been produced between species belonging to this family, for example *Arabidopsis thaliana* (+) *Brassica campestris* (Gleba and Hoffman 1980), *B. campestris* (+) *B. oleracea* (Schenk and Röbbelen 1982; Jourdan et al. 1986; Sundberg and Glimelius 1986; Terada et al. 1987), *Moricandia arvensis* (+) *B. oleracea* (Toriyama et al. 1987a), *Sinapis turgida* (+) *B. oleracea* and *S. turgida* (+) *B. nigra* (Toriyama et al. 1987b). If these hybrids are to be evaluated for practical plant breeding, they must be fertile, as some of them are (Schenk and Röbbelen 1982; Jourdan et al. 1986; Sundberg et al. 1987).

The present investigation describes the production of intergeneric somatic hybrids via protoplast fusion between *Brassica napus* and *Eruca sativa*. Ploidy level, fertility and chloroplast genotype of the hybrids were investigated. Since *E. sativa* is tolerant to drought and aphids (Tsunoda et al. 1980), traits from this species are of agricultural importance and of interest for transfer to *B. napus*.

Material and methods

Plant material

As parental material, *Brassica napus* L. ssp. *oleifera* cv 'Hanna' and *Eruca sativa* Mill. were used. Seeds were kindly provided from W. Weibull AB, Sweden and the Botanical Garden, Uppsala, Sweden.

Protoplast isolation, fusion and selection

Protoplasts of *B. napus* were isolated from 5-day-old hypocotyls. From *E. sativa*, protoplasts were isolated from leaves of in vitro grown plants approximately 3 weeks old. The culture of plant material and protoplast isolation were performed according to Glimelius (1984). Protoplast fusion, culture and treatment before selection was the same as that described by Glimelius et al. (1986) and Sundberg and Glimelius (1986).

Hybrid cells were selected with a micromanipulator (Sundberg and Glimelius 1986) or by flow cytometry and cell sorting (Glimelius et al. 1986).

Cell culture

Cells were cultured in a modified 8p medium (Glimelius 1984), where the initial hormone concentrations were as reported in Table 1. After 3–6 days, the cells were diluted with new culture medium (without hormones) to 4 times the original volume. When small cell aggregates could be observed (12–14 days after feeding), the colonies were transferred to modified K_3 medium (Menczel et al. 1981) containing 0.13% agarose and the same hormone concentrations as obtained after feeding (¼ of the initial hormone concentration, except in one case, see Table 1). When the calli had reached a diameter of 2–3 mm, they were transferred to modified K_3 medium with 0.4% agarose containing 0.6 μ*M* indole-3-acetic acid (IAA) together with 6-benzylaminopurine (BAP) and zeatin at various concentrations (2.2–9.1 μ*M*). The calli were transferred to fresh media every second week. Light and temperature conditions during the culture period were as previously described by Sundberg and Glimelius (1986). Shoots emerging from the calli were transferred to MS medium (Murashige and Skoog 1962) for rooting. The shoots were cultured under sterile conditions in climate chambers (25 °C, daylength 16 h under warm-white fluorescent light, 35 W/m^2 and 40 W tungsten lamps).

Table 1. Hormone concentrations during different stages of the culture period of fused cells obtained after protoplast fusion between *B. napus* and *E. sativa*

Experiment	Hormone concentration (μ*M*)					
	Initial			After plating		
	2,4-D[a]	BAP[b]	NAA[c]	2,4-D	BAP	NAA
1	2.2	0.55	0.5	0.55	0.14	0.12
2	2.2	0.55	0.5	0.55	0.14	0.12
3	0.45	0.55	0.5	0.11	0.14	0.12
4	4.5	2.2	0.5	0.4	0.55	0.12
5	4.5	2.2	0.5	1.12	0.55	0.12
6	4.5	2.2	0.5	1.12	0.55	0.12

[a] 2,4-dichlorophenoxyacetic acid
[b] 6-benzylaminopurine
[c] 1-naphthylacetic acid

Analysis of isozymes, nuclear DNA content and chloroplast genotype

Isozyme analyis was performed according to Sundberg and Glimelius (1986). The nuclear DNA content of the hybrids was determined in a flow cytometer as described by Fahleson et al. (1988). Chloroplast DNA (cpDNA) was isolated and digested, and the fragments separated by electrophoresis according to Sundberg et al. (1987).

Pollen viability and seed set

Pollen viability was investigated using the methods described by Sundberg et al. (1987). Seed set for each hybrid was recorded after self-pollination and backcrossing to the parental species. In each hybrid, 50 flowers were self-pollinated, and 50 flowers were pollinated with pollen from *B. napus*. Fertility was then expressed as seeds per pollinated flower in percentage of self- or cross-pollinated *B. napus*.

Results

Cell culture and shoot regeneration

The results from the fusion experiments are summarized in Table 2. In the experiments where a micro-manipulator was used to select hybrid cells, the plating efficiency was on average 9.5%. For the experiments where flow sorting was used as the selection method, the plating efficiency was on average 5.9% (see Table 1 for hormone concentrations in the different experiments). A majority of the shoots (80%) were regenerated under the following hormone regimes: 2.2 μ*M* BAP, 2.3 μ*M* Zeatin and 0.6 μ*M* IAA or 9.1 μ*M* Zeatin and 0.6 μ*M* IAA. Figure 1 shows hybrid plants in the greenhouse at the time of flowering.

Isozyme analysis

Of 8 different isozymes tested, 3 enzymes, leucine aminopeptidase (LAP), phosphoglucose isomerase (PGI) and 6-phosphogluconate dehydrogenase (6-P) had species-specific zymogram patterns (Fig. 2). When analysing the isozyme PGI, the uppermost parental isozyme band of *E. sativa* was usually very faint and hardly detectable in the hybrids, which was why this band was excluded as marker. In the case of 6-P, both the upper and lower regions of enzyme activity could be used as markers (Fig. 2c and d). Of the 65 shoots obtained, 54 were investigated (11 died at an early stage), and 34 were confirmed to be hybrids. Of these, 23 that were derived from different calli have up to now been successfully transferred to the greenhouse. The result of isozyme analysis of these 23 hybrids is shown in Table 3. In those cases where no hybrid isozyme pattern was found the zymogram was identical to *B. napus*. Eleven out of twenty-two hybrids (50%) showed the hybrid isozyme pattern for only one or two of the three investigated isozymes (Table 3).

Table 2. Results from protoplast fusion experiments between *B. napus* and *E. sativa*

Experiment no.	Fusion frequency[a] (%)	Selection method[b]	No. of selected cells	Hybrid cell frequency after selection (%)	Plating efficiency[c] (%)	No. of shoots from different calli	Regeneration frequency[d] (%)
1	9.7	MM	310	100	11.3	2	5.7
2	11.1	MM	240	100	8.3	–	–
3	9.1	MM	240	100	8.3	–	–
4	7.1	FCM	6000	80	11.3	6	0.9
5	6.0	FCM	4000	58	12.8	4	7.8
6	nd	FCM	6000	57	5.0	53	17.7

[a] Calculated at the day of fusion
[b] MM = Micro-manipulator, FCM = flow cytometer
[c] In percentage of the number of plated calli divided by the number of selected cells
[d] Shoots obtained as percentage of plated calli
nd = not determined

Table 3. DNA content, isozyme pattern, fertility, flower design and chloroplast genotype of the hybrids transferred to the greenhouse

Hybrid no.	DNA content	Isozyme analysis[b]			Pollen viability[c]	Seed set[d]		Flower colour	Petal shape	Chloroplast genotype[e]
	pg/nucleus	LAP	PGI	6-P		Selfed	× B.n.			
J 1	3.28	+	+	+	57.9	3.8	23.9	Yellow	Intermediate	*B. napus*
J 4	2.95	+	+	+	28.9	3.0	21.1	Pale yellow	*B. napus*	*E. sativa*
J 5	2.65	–	+	+	68.4	3.7	74.9	Pale yellow	Intermediate	*B. napus*
J 6	2.80	+	+	+	43.4	3.8	31.5	Pale yellow	Intermediate	*B. napus*
J 8	2.53	+	nd	–	67.1	28.8	55.6	Yellow	Intermediate	nd
J 9	4.03	+	+	+	31.6	0	0.2	Pale yellow	Intermediate	nd
J10	3.24	+	+	+	48.7	0.5	5.2	Pale yellow	*B. napus*	*B. napus*
J12	2.71	+	nd	nd	19.7	2.0	27.7	Pale yellow	*B. napus*	nd
J13	5.15	+	–	–	18.4	0.2	1.6	Yellow	Intermediate	*B. napus*
J14a	4.45	+	–	+	30.2	0.3	2.8	Pale yellow	Intermediate	*E. sativa*
J15	4.97	+	+	–	26.3	4.1	1.1	Yellow	Intermediate	*B. napus*
J17	2.64	+	+	+	38.1	1.8	14.2	Pale yellow	Intermediate	*B. napus*
J18	2.71	+	+	+	52.6	15.8	11.9	Pale yellow	*B. napus*	*B. napus*
J19	3.15	+	+	+	98.7	1.4	48.7	Pale yellow	Intermediate	*B. napus*
J21	2.74	–	+	nd	8.2	0	0	Pale yellow	Intermediate	nd
J23	2.96	–	+	–	31.6	1.9	17.4	Yellow	Intermediate	*B. napus*
J25	4.90	+	–	–	died					
J26	2.82	–	+	–	59.2	9.9	36.9	Yellow	*B. napus*	*B. napus*
J27	5.08	+	+	+	11.8	1.3	1.6	Pale yellow	Intermediate	nd
J14b[a]	2.57/5.26	+	nd	+	8.8	0.3	5.8	Pale yellow	Intermediate	nd
J29	5.22	+	+	+	14.5	0.5	2.9	Pale yellow	*B. napus*	nd
J30	3.01	+	+	+	14.5	2.0	186.0	Pale yellow	*B. napus*	nd
J31	3.50	+	+	–	27.6	4.5	40.5	Pale yellow	Intermediate	nd
J32	4.82	–	+	+	died					
Parental material										
B. napus	2.10					7.9[f]	9.4[f]	yellow		
E. sativa	1.30							white		

[a] Originally a cloned copy from J14a
[b] A plus sign (+) denotes that isozyme bands from both parents are present in the hybrid, a minus sign (–) denotes that only bands from *B. napus* are present
[c] As % of *B. napus*
[d] As % of self- or cross-pollinated *B. napus*
[e] According to restriction fragment pattern after digestion with Bam HI
[f] Values represents the number of seeds obtained per pollinated flower
nd = not determined

Fig. 1. Hybrid plants in the greenhouse at the time of flowering

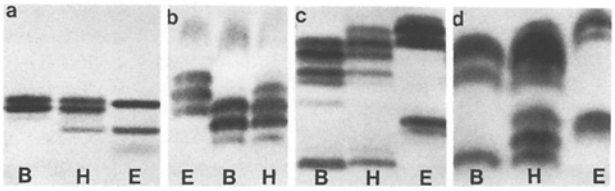

Fig. 2a–d. Sections of isozyme gels stained for **a** LAP (monomeric enzyme), **b** PGI (dimeric enzyme), **c** and **d** 6-P (dimeric enzyme). *B*, *B. napus*; *H*, hybrid; *E*, *E. sativa*

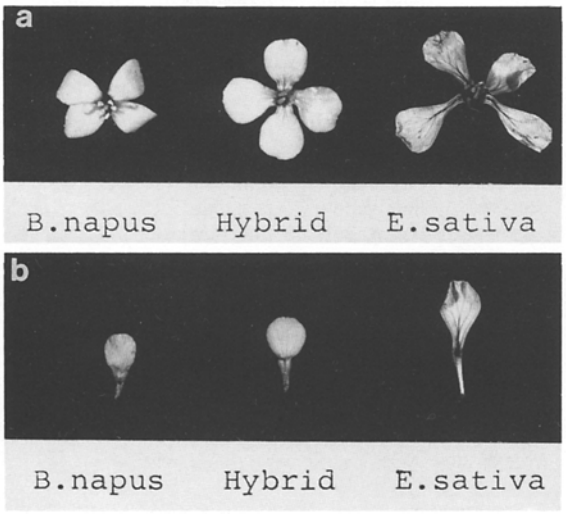

Fig. 3. **a** Flowers from one of the hybrids compared to flowers from the parental species. **b** Petals from one of the hybrids compared to petals from the parental species

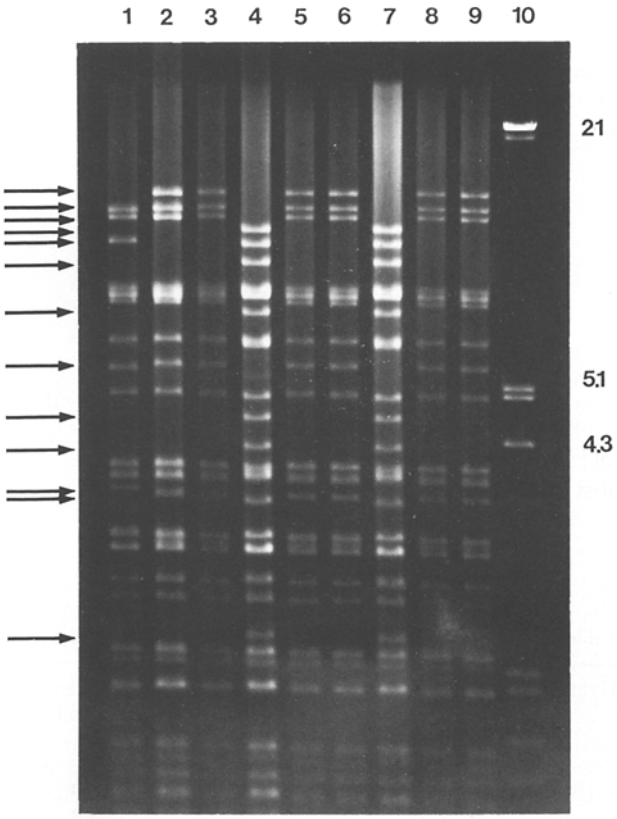

Fig. 4. Restriction fragment pattern of chloroplast DNA from parental and somatic hybrid plants after digestion with BamHI restriction endonuclease. *Lane 1*, *B. oleracea*; *lane 2*, *B. campestris*; *lane 3*, *B. napus*; *lane 4*, *E. sativa*; *lane 5*, J1; *lane 6*, J5; *lane 7*, J14a; *lane 8*, J15; *lane 9*, J19; *lane 10*, molecular weight standard lambda DNA cut with EcoRI/HindIII. The differences in restriction patterns between the parents are indicated with *arrows*

Analysis of nuclear DNA content, pollen viability, seed set and morphological characters of the hybrids

As presented in Table 3, the nuclear DNA content varied from 2.4 to 5.3 pg DNA/nucleus for the different hybrids. One of the hybrids contained 2 types of cells that differed in DNA content (J14b). Pollen viability was on average 45.2% of that of *B. napus*, although a large variation (14.5%–98.7%) was observed. Fertility was estimated as seeds per pollinated flower. Fertility after self-pollination was on average 7.0% of that of self-pollinated *B. napus*. Here as well, a large variation, 0%–28.8%, was noted. In backcrosses to the parents, only crosses to *B. napus* gave seeds. The fertility was on average 26.4% (range 0%–186%) of the fertility of cross-pollinated *B. napus*. Flower colour and petal morphology were studied (Fig. 3, Table 3): of 21 hybrids from different calli, 20 were intermediate for at least one of the characters. Where no intermediate character was found, the flower colour or petal shape resembled *B. napus*.

Analysis of the chloroplast genotype of the hybrids

Of 13 investigated hybrids, 11 showed the chloroplast restriction fragment pattern of *B. napus*, while only 2 had the pattern of *E. sativa* (Table 3, Fig. 4). The chloroplasts of *B. napus* cv 'Hanna' had a restriction fragment pattern identical to *B. campestris* (Fig. 4).

Discussion

The present investigation clearly demonstrates that protoplast fusion can be used as an efficient alternative for production of the intergeneric hybrid between *B. napus* and *E. sativa*. These hybrids can be produced by sexual crossing, but at the low frequency of 0.002 hybrids per pollinated flower (Downey et al. 1980). Furthermore, almost all hybrids in the present study had some degree of fertility and produced seeds, making them useful for plant breeding purposes.

Selecting hybrids with a micro-manipulator is very accurate and makes it possible to select and culture pure populations of hybrids (Sundberg and Glimelius 1986). This enables studies of cell division and plating efficiency of the fusion products. In the present investigation, three experiments were carried out using a micro-manipulator, and an average plating efficiency of 9.5% was noted. This is in good agreement with the interspecific hybridization made between *B. campestris* and *B. oleracea* (Sundberg and Glimelius 1986) and indicates that in the present study, early cell division and cell growth were not inhibited in spite of the taxonomic distance between the species. For the regeneration of plants, the scheme developed for *B. napus* (Glimelius 1984) was followed. In our study, a large variation in regeneration frequency was observed which could be due either to inadequate culture conditions or to genomic imbalances between the combined species.

As shown by Sundberg and Glimelius (1986), isozyme analysis is a suitable method for identifying hybrids between species belonging to the *Brassicaceae* family. From isozyme analysis, it was shown that 50% of the investigated hybrids were partial (asymmetric hybrids). The results furthermore indicate that the hybrids preferentially had lost chromosomes from *E. sativa*. This conclusion was based on the fact that when a partial hybrid displayed an isozyme pattern from only one parent for a particular isozyme, this pattern was identical to *B. napus*. Preferential loss of chromosomes from one of the species combined in a hybrid cell by protoplast fusion has been documented in other investigations (Kao 1977; Pental et al. 1986; Pijnacker et al. 1987).

A large variation in nuclear DNA content was found in the hybrids. The analysis of DNA content also indicated that the hybrids were nuclear hybrids, since all except one (J14b) generated a single peak of fluorescence. However, a majority of the hybrids deviated from the sum of the parental DNA values. Taken together, the analysis of DNA content and the isozyme analysis suggest that for those hybrids confirmed to be asymmetric by isozyme analysis, but exceeding the sum of the parental DNA values, the sorting out of *E. sativa* chromosomes from the hybrid calli was accompanied by a duplication of chromosomes (predominantly *B. napus* chromosomes). This could explain the high DNA value in connection with the fact that these hybrids always contained zymograms identical to the *B. napus* type when no hybrid isozyme pattern was found. A duplication of chromosomes could be attributed to the cell culture period since, as reported in several investigations (Larkin and Scowcroft 1981; Newell et al. 1984), regeneration of plants via a callus culture phase often results in a certain amount of plants with an abnormal chromosome number. Another possible explanation for the presence of asymmetric hybrids with high DNA values could be that for each of these hybrids a multifusion event occurred, followed by a sorting-out of chromosomes (preferentially *E. sativa* chromosomes).

In spite of the fact that many of the hybrids were asymmetric, almost all hybrids were fertile to some extent and produced seeds, making them useful for practical plant breeding purposes. Generally, as expected, the seed set was higher when the hybrids were backcrossed to *B. napus* than when they were self-pollinated, owing to the lower viability of hybrid pollen compared to *B. napus* pollen.

The restriction fragment pattern of chloroplast DNA (cpDNA) was studied in the parental species and in 13 hybrids. The pattern of *B. napus* cpDNA was identical to that of *B. campestris*, indicating that the chloroplasts of *B. napus* cv 'Hanna' were inherited from *B. campestris*. As expected, the chloroplast restriction fragment pattern of *B. napus* was rather similar to that of *B. oleracea*, but very different from that of the more distantly related *E. sativa*. A majority of the investigated hybrids had a chloroplast restriction fragment pattern identical to that of *B. napus (B. campestris)*. This indicates a non-random segregation of chloroplasts and could be due to several factors, such as different rates of replication as discussed by Bonett and Glimelius (1983) or plastome-genome incompatibilities.

In summary, this investigation shows that a large number of somatic hybrids between *B. napus* and *E. sativa* can be obtained from only a few experiments. Although a majority of the hybrids were found to be asymmetric, no severe problems concerning the fertility could be observed among them. Somatic hybridization is obviously an efficient method for transferring genetic material from *E. sativa* to *B. napus*.

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