Theor Appl Genet (1995) 91: 236–241

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Inheritance of rapeseed (*Brassica napus*)-specific RAPD markers and a transgene in the cross *B. juncea* × (*B. juncea* × *B. napus*)

Received: 14 December 1994 / Accepted: 17 January 1995

Abstract We have examined the inheritance of 20 rapeseed (*Brassica napus*)-specific RAPD (randomly amplified polymorphic DNA) markers from transgenic, herbicide-tolerant rapeseed in 54 plants of the BC_1 generation from the cross *B. juncea* × (*B. juncea* × *B. napus*). Hybridization between *B. juncea* and *B. napus*, with *B. juncea* as the female parent, was successful both in controlled crosses and spontaneously in the field. The controlled backcrossing of selected hybrids to *B. juncea*, again with *B. juncea* as the female parent, also resulted in many seeds. The BC_1 plants contained from 0 to 20 of the rapeseed RAPD markers, and the frequency of inheritance of individual RAPD markers ranged from 19% to 93%. The transgene was found in 52% of the plants analyzed. Five synteny groups of RAPD markers were identified. In the hybrids pollen fertility was 0–28%. The hybrids with the highest pollen fertility were selected as male parents for backcrossing, and pollen fertility in the BC_1 plants was improved (24–90%) compared to that of the hybrids.

Key words *Brassicaceae* · Interspecific hybridization · Synteny groups · Risk assessment

Introduction

Brassica juncea is cultivated in Asia, USA and Canada especially for oil and mustard production. In Denmark and Sweden the species is found as a weed or ruderal; in Southern Europe it is naturalized (Hultén and Fries 1986; Mossberg et al. 1992; Heywood and Akeroyd 1993). The close relationship between *B. juncea* and rapeseed (*Brassica napus*) calls for investigations of the genetics of species' hybrids and subsequent backcross generations. One question to address is if the future cultivation of transgenic rapeseed will bring about dispersal of the transgene to wild *B. juncea*.

Prerequisites for gene dispersal from one species to another are that the species can hybridize and backcross and that alien genes are subsequently stably integrated in the new genetic background. Hybridization between *B. napus* and *B. juncea* in controlled crosses is easy (Heyn 1977; Prakash and Chopra 1990). *B. juncea* is self-compatible; however, in the field between 3% and 30% of the seeds are derived from outcrossing (Voskresenskaya and Lygina 1973), and outcrossing can bring about spontaneous interspecific hybridization between *B. juncea* and *B. napus* (Bing et al. 1991).

Genome analysis has shown that the diploid *B. campestris* (2n=20, genomic composition AA) is one of the progenitors of the two amphidiploid species *B. napus* (2n=38, AACC) and *B. juncea* (2n=36, AABB) (U 1935). Hybrids from crosses between *B. napus* and *B. juncea* usually form bivalents between the A-chromosomes (U 1935; Prakash and Hinata 1980). This suggests a high degree of homology between the A-genomes of the two species and that an exchange of genes, located on the A-genomes, can occur relatively unimpeded by recombination. Studies of interspecific hybrids and marker analysis have revealed substantial homology between the A- B- and C-genomes (U 1935; Prakash and Chopra 1990; McGrath and Quiros 1991; Quiros et al. 1994. Therefore, intergenomic recombination may occur and provide introgression to *B. juncea* of genes located on the C-genome of *B. napus*. Introgression to *B. juncea* could also be accomplished by C-chromosome additions (Lee and Namai 1992) or substitutions.

In plants from the selfing of the trigenomic hybrid *B. napus* × *B. campestris*, a non-random distribution of isozyme markers specific to the C-genome has been observed, with the frequency of C-genome markers ranging from 0% to nearly 100% (Chen et al. 1990). A screwed distribution of C-genome synteny groups was also reported in backcross combination (*B. napus* × *B. campestris*) × *B. campestris* (McGrath and Quiros 1990). This offers a possibil-

Communicated by P. M. A. Tigerstedt

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ity to pinpoint the parts of the C-genome which are rarely dispersed to *B. campestris*, and preferably a transgene should be integrated in these genome regions. If a non-random distribution of genetic material from rapeseed also takes place in the cross *B. juncea* × (*B. juncea* × *B. napus*), it should be taken into consideration when producing and selecting transgenic *B. napus*. The inheritance of rapeseed-specific markers in crosses with *B. juncea* is analyzed in this study.

In this article we report on the inheritance of 20 *B. napus*-specific randomly amplified polymorphic DNA (RAPD) markers and a transgene from rapeseed in the BC_1 generation of the cross *B. juncea* × (*B. juncea* × *B. napus*).

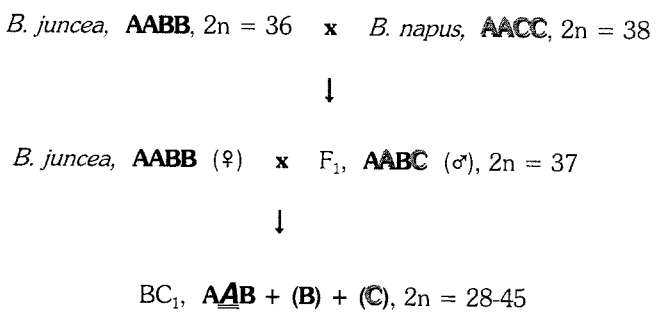

Fig. 1 Crossing scheme. *Ordinary fount B. juncea, shaded fount B. napus, underlined fount* recombined genome. *Brackets* indicate that only a part of the genome may be present. The chromosome numbers of the genomes are: *A* n=10, *B* n=8, *C* n=9

Materials and methods

Plant material

The species specificity of the rapeseed RAPD markers was examined. One plant from each of the following lines of rapeseed was tested: 11-7060 ('Loras' × 'Karat'), 11-7094 ('Karat' × 'Tower'), 'Line', 'Topas' and six transgenic lines containing a gene construct providing tolerance to the herbicide glyphosat, 9110090, -092, -096, -121 ('Karat' × 'Loras'), 111251 and -252 ('Westar'). The transgenic lines were obtained from Maribo Seed, Holeby, Denmark and Monsanto Company, Missouri. The rapeseed specificity of the amplification products was confirmed by comparing them with RAPD profiles from a total of 32 *B. juncea* plants from two different accessions (20 individuals from no. 91, The Botanical Garden, University of Uppsala, Sweden, and 12 individuals from no. 872, The Botanical Garden, University of Copenhagen, Denmark; collected at Jersie Strand, Denmark).

The parentals used in the crosses were among the plants analyzed. All six transgenic *B. napus* lines were crossed with plants from both *B. juncea* accessions. Seeds were obtained from all 12 genotypic combinations and germinated on filter paper in petri dishes. Plantlets were potted in a growth chamber (16 h light, 22 °C/8 h dark 18 °C) at the cotyledon stage.

Crossing technique and scheme

Young flowers of the female parent were emasculated and covered with a parchment bag. After 3 days pollen was applied to the buds, and they remained covered until the development of siliquas.

The crossing scheme is shown in Fig. 1, and Table 1 presents the number of flowers pollinated in the crossing combinations. The inheritance of the RAPD markers was analyzed in two BC_1 families: the two transgenic *B. napus* lines 9110092 and -121 and the *B. juncea* accession no. 91 were used for producing these backcross plants (Table 1). The *B. napus* lines had one copy of the transgene (J. Brunsted, Danisco Biotechnology, Copenhagen, Denmark, personal communication). From each of the two *B. napus* lines 1 plant was crossed with 1 *B. juncea* plant, and the seeds were harvested at maturity. Twenty randomly chosen F_1 seeds from each crossing combination were germinated, and the resulting hybrids were investigated for pollen fertility and presence of the transgene. From each crossing combination the transgenic hybrid with the highest pollen fertility was selected as pollen donor in the backcross to *B. juncea*. The same *B. juncea* plant was used as the female in all backcrosses (Table 1).

Pollen fertility

Pollen fertility was estimated by staining with cotton-blue (Phillips 1981). From each plant 400 pollen grains from a minimum of two flowers were analyzed.

PCR and RAPD

Two different DNA extraction techniques were used. DNA was extracted from 3 g of young leaves by the method of Saghai-Maroof et al. (1984) using the modifications recommended by Poulsen et al. (1993) and a ribonuclease A treatment (10 l/ml, 37 °C for 30 min) preceding precipitation with isopropanol. During the project a simpler method by Edwards et al. (1991) was introduced and applied to the F_1-generation. The two different extraction methods were compared by RAPD analyses, and no differences were found with respect to the RAPD markers in use. From each plant in the BC_1 generation two individual extractions were made; one by the method of Saghai-Maroof et al. (1984), with the modifications described above, reduced 1:30, and one by the method of Edwards et al. (1991). The leaf material for each extraction, a total amount of approximately 1.5 cm^2, was picked from five different leaves of the plant.

Oligonucleotide primers specific to the transgene providing tolerance to glyphosat (patent by Monsanto Company) were produced by DNA Technology (Forskerparken, Århus, Denmark). Primers for the RAPD technique were from Operon Technologies (Alamedea, Calif.). The temperature cycle in the polymerase chain reaction (PCR) was as follows: 94 °C for 5 min; 45 cycles of 93 °C for 2 min, 55 °C for 2 min and 72 °C for 3 min; 72 °C for 10 min and finally 30 °C for 1 s. Temperature cycles and concentrations of the constituents in the RAPD reaction were according to Williams et al. (1990). The amplifications were carried out on a Hybaid Omnigene with Super *Taq* polymerase from HT Biotechnologies (Cambridge, UK). A volume of 16 µl of amplification product was added to 4 µl loading buffer II and analyzed by electrophoresis in 1.4% agarose gels with ethidium bromide (Sambrook et al. 1989). Bands were visualized by UV light.

The amplification products of the RAPD and PCR analysis were scored by their presence or absence. Results of the assays on the BC_1 generation represent a consensus of two replicates with two individual DNA extractions of each plant, as described above.

Data hypothesis, -treatment and -analysis

A 50% transfer of a genetic marker to the BC_1 generation should be expected if (1) the marker is present in only one chromosome, (2) the *B. napus* plant is homozygous at the marker locus, (3) the *B. napus* chromosome with the marker locus is maintained during all cell divisions in the hybrid and the BC_1 plants, and no chromosome deletions occur, (4) the chromosomes are randomly distributed during meiosis, and inter- and intragenomic recombination is rare, (5) selection against specific genotypes does not take place. If we assume that the marker is present in two chromosomes instead of one, it will be found in 75% of the BC_1 generation. These two hypotheses (marker present at one chromosome contra two chromosomes) were tested by a χ^2 comparison ($P < 0.05$). For cluster analysis, χ^2 for associ-

Table 1 Results from crosses and backcrosses (*B.j. B. juncea*, *B.n. B. napus*)

Crossing combination	Flowers pollinated	Average seed set[a]	Germination (%)	Pollen fertility (%)
B.j. (♀) × *B.n.* (♂)	170	4.6	93	0–31[b]
B.n. (♀) × *B.j.* (♂)	120	0.18	4.5	–
for RAPD analysis:				
B.j.–1 (♀) × *B.n.*092–1 (♂)	16	6.9	100	0–24
B.j.–2 (♀) × *B.n.*121–1 (♂)	19	3.3	90	0–28
B.j.–3 (♀) × (*B.j.*–1 (♀) × *B.n.* 092–1 (♂))	105	1.1	75	24–82
B.j.–3 (♀) × (*B.j.*–2 (♀) × *B.n.* 121–1 (♂))	51	0.65	74	32–90

[a] Average seed set is the average number of seeds per pollination
[b] A total of 100 hybrids were investigated, 2–14 from each crossing combination

ation of pairwise segregation of markers was used to construct dendrograms by the method of nearest neighbor and the average method (Pimentel 1979). The synteny groups were accepted if indicated by both methods.

Results

Hybridization and backcrossing

Results from the crosses are presented in Table 1. The hybridization between *B. napus* and *B. juncea* was found to be most effective when *B. juncea* was used as the female parent. The average number of F_1 seeds per pollination was 4.6 with *B. juncea* as the female, which was considerably more than that obtained in the reciprocal cross (0.18 seeds per pollination). The seeds harvested on *B. napus* were either often deformed with no embryo or they started to germinate in the siliquas. The two hybrids with the highest pollen fertility were used as male parents in the backcrosses. These plants had a pollen fertility of 24% (*B. juncea* × 9110092) and 28% (*B. juncea* × 9110121). The backcrosses produced 148 seeds with a germination percent of 75% compared to 90–100% for the initial cross. Fifty-four randomly picked BC_1 plants were analyzed by the RAPD technique.

In a small field experiment where *B. juncea* plants were transplanted into a *B. napus* field, 3% of the seeds harvested on the *B. juncea* plants were *B. napus* hybrids (unpublished data). This frequency corresponds to the 1–3% of interspecific hybrids obtained by Bing (1991) and Bing et al. (1991).

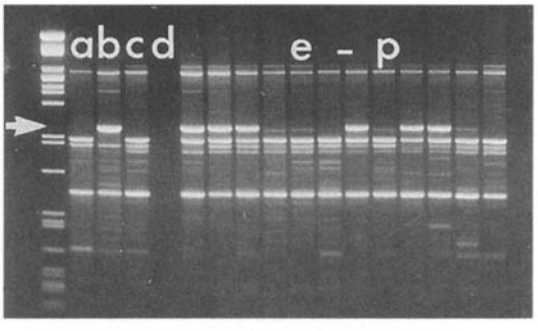

Fig. 2 Electrophoresis of amplification products produced with primer A17. *a B. juncea*, *b B. napus*, *d* negative control, *c* and *e-p* BC_1 plants. The *arrow* indicates the position of the marker A17-1300

Segregation of RAPD markers and the transgene

From a survey of several RAPD primers, 13 selected primers produced a total of 20 *B. napus*-specific marker bands that were easily detectable and reproducible in the parental *B. napus* plants and the F_1 hybrids but not present in the *B. juncea* plants. Table 2 shows the segregation of these markers in the backcross generation. All of the *B. napus*-specific markers were found among the BC_1 plants analyzed, which showed 0–20 markers per plant (mean=13 markers per plant). Two plants had none of the markers; 1 plant had all 20 markers. One of the RAPD markers (A04-2000) was found in significantly less than 50% of the BC_1 plants, 10 markers were found in significantly more than

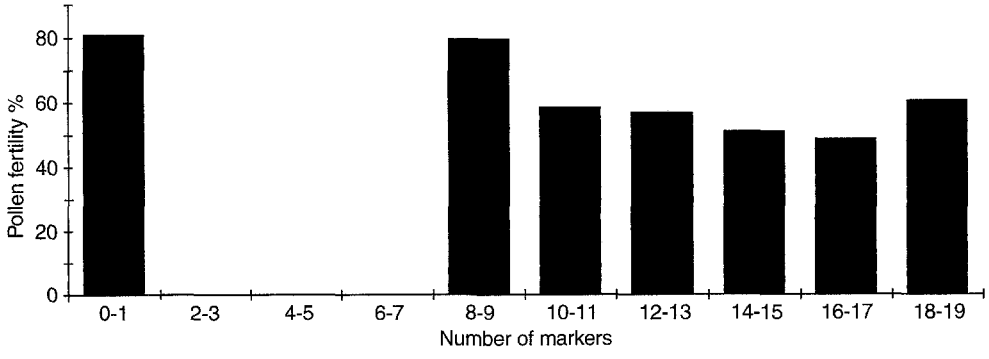

Fig. 3 Correlation between pollen fertility and number of RAPD markers in 28 random BC_1 plants

Table 2 Synteny groups, distribution of RAPD markers in the 54 BC_1 plants and frequencies of transfer of *B. napus*-specific RAPD markers and the transgene to the BC_1 generation (± the marker is present/absent, *?* the marker could not be scored, χ^2>3.85 (P< 0.05) indicates that the frequency of transfer of the marker is significantly different from 50% and 75%, respectively)

Synteny group	Marker	Distribution of markers in the backcross plants. Plant number:						Frequency of transfer (%)	χ^2 50%	χ^2 75%
		1–10	11–20	21–30	31–40	41–50	51–54			
	A04-850[a]	+−+−+++−−+	+−+−+++−−−	++++−+++++	+++−++++−−	−++++−+++−	+++−	70	8.96	0.617
	A04-2000[a, b]	−−+−−−−−−−	−−−−+−−−+−	−+−−+−−−+−	++−−−−+−−−	−−−−−−−−−−	−−−−	19	21.4	91.8
1	A09-750[b]	−−+−+++−++	++−−++−++−	−+−−+−−++−	+−+−+−++−+	+−++++−+−	−−−−	54	0.396	13.1
	A09-1000[b]	−−+++−−++−	++−−−+++−−	++++++−+++	−+−+−−++++	−+++−−+−++	++++	63	3.62	4.17
4	A11-300[b]	−−+−−−+−++	−−+−−++++−	?+++++−−++	++−−+++++−	+−+++−++++	++−−	62	3.18	4.59
5	A11-400[a, b]	−−+++++++++	+++−++++−−	++++++++++	++++++++++	++++++++++	++++	93	39.2	8.91
1	A11-1200[b]	−−++−−−−++	+−−−++−++−	+−+−−−++?+	−+−+−++++−	−+−+−−+−+−	+−−+	49	0.02	21.5
5	A13-700[a, b]	+−++++++++	+++−++++−−	++++++++++	++++++++++	++++−++++	++++	93	39.2	8.91
1	A16-1700[b]	−−+++−−−−+	++−−−++−−−	++++++−+++	−+−+−++++	−++−−−+−+−	++++	59	1.85	7.14
	A17-1300[a]	−−+++−−−++	++−−+++−+−	++++++++++	++−+++++++	+++++++++−	−+++	78	16.7	0.222
3	B05-900[b]	+−−−++−−−−	−−−+−−+++−	−++−++++−−	++−+++−+++	−+−+−−−−−−	−+−−	44	0.667	26.9
4	C06-350[a]	+−+++−−−++	++−−++++−−	+++++−+++	++−+−+++++	+++++−+−++	++++	78	16.7	0.222
	C06-900[a]	−?+−−−+−++	−−+−−++++−	+++++−+++	++−−++++−−	+++++−++++	+++−	70	8.32	0.761
3	C06-1400[b]	+−+−++−−−−	−−−−−−+++−	+++++−−+−	++−+++−+++	−+−+−−−−−−	−++−	48	0.074	20.8
	C07-1400[a]	−−++−−+−++	+++−+++−+−	+++++−−+++	++++++++++	+++++−++++	++−	76	14.5	0.0247
	C09-480[a]	−−++++++−?	+++−+++−+−	+++++−−+++	++++−+++++	++++++++++	++++	85	25.8	2.77
2	C13-780[b]	+−++−+++−−	−+−−−++−+−	+++++−+−+−	−+−+−+−+−−	−+−−++−−−−	++++	52	0.074	15.4
2	C13-850[b]	+−++−+++−−	−+−−−++−−−	+++++−+−?−	−+−+−+−+−−	−+−−++−−−−	−+++	47	0.170	21.9
	C14-1400[a]	+−++−+++−+	−−−−−++++−	++−+−+++++	+++−+−−++−	++++++++++	+++−	70	8.96	0.617
	F09-850[a]	+−++++++++	+++−++++−−	++++−++++	+++−++−+−+	++++−−++++	−+++	80	18.9	0.617
	Total							65		
	Transgene									
	B.j.×B.n.092	+ +−+−++−+	++−?	?−−				57	0.25	3.0
	B.j.×B.n.121[b]	−	+?+−+−	−−++??	−+			46	0.09	4.12

[a] Markers with a frequency of transfer significantly different from 50%
[b] Markers with a frequency of transfer significantly different from 50%

50% of the BC_1 plants and 2 of these markers were found in significantly more than 75% of the BC_1 plants. The remaining 9 RAPD markers had a frequency of transfer not significantly different from 50%. Figure 2 shows the segregation of marker A17-1300 in some backcross plants. Due to technical difficulties the frequency of inheritance of the transgene was estimated on the basis of only 27 BC_1 plants. The transgene was found in 14 (=52%) of these plants with apparently no significant difference in inheritance between the two backcross families.

Pollen fertility of BC_1 plants

Maximum pollen fertility among the hybrids was found to be 28%. The pollen fertility of BC_1 plants were significantly higher, ranging from 24–90% (Table 1). Apparently, the BC_1 plants with only a few RAPD markers had the highest frequency of viable pollen (Fig. 3). However 3 of the transgenic BC_1 plants with many RAPD markers had a high pollen fertility: Two plants showed 19 of the 20 RAPD markers and a pollen fertility of 70%, and 1 plant with 17 RAPD markers present had a pollen fertility of 74%. Most BC_1 plants were able to produce seeds in open pollinations in the greenhouse.

Synteny groups

Synteny groups should be identified cautiously when the markers show deviations from a Mendelian segregation. However, in the cluster analysis the actual segregation of the markers was taken into account. Five synteny groups were identified by means of both the method of nearest neighbor and the average method (Table 2): synteny group 1, A09-750, A11-1200, A16-1700; synteny group 2, C13-780, C13-850; synteny group 3, B05-900, C06-1400; synteny group 4, A11-300, C06-350; and synteny group 5, A11-400 and A13-700. The support in favor of linkage between the markers of each synteny group is good (probability for independence is less than 1%).

Discussion

We have confirmed that interspecific hybrids between *B. napus* and *B. juncea* are easy to obtain in controlled crosses with *B. juncea* as female parent, and we found that spontaneous interspecific hybridization is also possible. With one exception, marker A04-2000, we found no indication of selection against *B. napus*-specific markers in the backcross generation from the cross *B. juncea* × (*B. juncea* × *B. napus*).

Because *B. juncea* was used as the female part in both hybridization and backcrossing, no cytoplasmatic genetic material from rapeseed was transferred to the BC_1 generation and the rapeseed RAPD markers were coded for by nuclear DNA sequences. Meiosis in the involved species reveals a regular bivalent formation (Prakash and Hinata 1980; Attia and Röbbelen 1986) and, therefore, the genomic constitutions of the plants involved in our crosses are as given in Fig. 1.

According to the hypothesis presented in the Materials and methods, we expected to find each marker in 50% of the plants of the BC_1 generation. In contrast to this expectation a frequency of transfer significantly higher than 50% characterized half of the RAPD markers used in our analysis. The most obvious explanation for this phenomenon is that these markers are present in the *B. napus* genome at more than one chromosome. One possibility is that the markers are expressed by both the A- and the C-genome. Quiros et al. (1991, 1994) found a high homology between the A-, B- and C-genomes on the basis of RAPD analysis. From restriction fragment length polymorphism (RFLP) data Derek Lydiate (John Innes Centre, Norwich, UK; personal communication) concluded that the differences between the A- and C-genomes of *B. napus* are mainly due to gene organization rather than to the number or nature of the genes present. Two of the markers (A11-400 and A13-700) were transferred to significantly more than 75% of the BC_1 plants. The very high frequency of these markers could be explained by the presence of more than two unlinked loci or to selection in favor of genes linked to these markers. Extensive gene duplication has been found within the A- and C-genomes (Coulthart and Denford 1982; McGrath et al. 1990; Kianian and Quiros 1992; McGrath and Quiros 1991; Quiros et al. 1994). Therefore, it is likely that some of the RAPD markers could be present at several unlinked loci.

Because of the possibility of intergenomic recombination, which has been reported by Quiros et al. (1987) and Chen et al. (1990), we cannot decide whether the identified synteny groups represent A- or C-chromosomes. Markers apparently linked in the same synteny group could also be distributed on different chromosomes, which for some reason are inherited together. Hu and Quiros (1991) and Quiros et al. (1994) have detected deletions in alien C-genome chromosomes in *B. campestris* monosomic addition lines (2n=20A+1C). Hu and Quiros reported that 46–56% of the progeny from two monosomic addition lines showed deletions in the alien C-chromosome. If certain of our markers are liable to be deleted, this might complicate their assignment to the linkage groups.

Risk assessment in relation to dispersal of transgenes from rapeseed to *B. juncea*

Our data suggest that gene dispersal from rapeseed, *B. napus*, to *B. juncea* cannot be neglected. We base this conclusion on the following results: (1) the cross compatibility between the genotypes of *B. napus* and *B. juncea* used in our crosses was good, (2) all 20 investigated *B. napus*-specific RAPD markers were transmitted to the BC_1 generation of the cross *B. juncea* × (*B. juncea* × *B. napus*), (3) some BC_1 plants were found to have a high pollen fertil-

ity, even though they carried most of the investigated *B. napus*-specific RAPD markers and the transgene.

Recombination between homoeologous A-chromosomes from *B. napus* and *B. juncea* is expected as well as intergenomic recombination between the A- and C-genomes. Our results therefore indicate that the introgression of *B. napus* genes into the genome of *B. juncea* is possible. In addition hybridization between *B. napus* and *B. campestris* (Jørgensen and Andersen 1994) and between *B. campestris* and *B. juncea* (Anand et al. 1985; Banga 1986) is easy. This suggests that gene exchange between these three species could occur.

Acknowledgements Maribo Seed and Monsanto Company are acknowledged for delivering part of the plant material. We want to thank Bente Andersen for excellent assistance in the laboratory. Lars Landbo, Thomas Mikkelsen, Hanne Østergård (Risø National Laboratory) and Freddy Christiansen (Aarhus University) are acknowledged for commenting on the manuscript. This research was made possible by a grant from the Danish Committee on Biotechnology.

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