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Inheritance of oilseed rape (*Brassica napus*) RAPD markers in a backcross progeny with *Brassica campestris*

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Abstract Different cultivars/transgenic lines of oilseed rape (*Brassica napus*) were crossed (as females) with different cultivars/populations of *Brassica campestris*. All cross combinations produced seed, with an average seed set per pollination of 9.8. Backcrossing of selected interspecific hybrids (as females) to *B. campestris* resulted in a much lower seed set, average 0.7 seed per pollination. In the single backcross progeny where a large enough population (92 plants) was obtained for analysis, 33 *B. napus*-specific RAPD markers were investigated to determine the extent of transfer of oilseed rape genetic material into this population. Markers were transferred to the backcross generation with frequencies ranging from 26% to 91%. Almost all of the markers (30/33) were transferred in a frequency not significantly different from 50%. Analysis of the pairwise segregation of markers revealed that 23 markers could be assigned to six linkage groups, most probably reflecting six *B. napus* C-chromosomes. The presence of backcross plants with recombinant genotypes suggests that complex genetic processes can take place during interspecific hybridisation and backcrossing in these *Brassica* species. The implications of our results for the possible choice of integration sites of transgenes in oilseed rape are discussed.

Key words Interspecific hybridisation · Introgression · Gene flow · Risk assessment · Transgenic plants

Introduction

Since it became possible to modify plants genetically by introducing specific genes into their genome, this technology has been used extensively. A large number of plant species is now amenable to such genetic manipulation, and many genes coding for traits such as herbicide tolerance, pathogen resistance and resistance to environmental stress have been inserted into plants (Dale et al. 1993; Raybould and Gray 1993). A large number of transgenic plants of potential commercial value have been tested in field trials (Dale et al. 1993; Krattiger 1994), and the first products produced by this technology have been marketed (e.g. Kramer and Redenbaugh 1994).

The widespread growth of transgenic crops is, however, not without risks. In the discussion of possible ecological risks, two distinct types are often invoked: (1) that the transgenic plant itself becomes a weed, affecting natural and/or agricultural habitats and (2) that the transgene is transferred to other species (e.g. weeds) by hybridization and backcrossing, and that this causes problems in natural and/or agricultural habitats. The introgression of a transgene, or any other gene, from transgenic plants to related species is probably a multi-generation process. Risk assessment studies have usually focused on the ability of the species of interest to cross, on the fertility of the interspecific hybrids, and on the ability of the interspecific hybrids to backcross and/or self (Raybould and Gray 1993; Scheffler and Dale 1994). In the majority of cases, characterization of the actual transfer of genetic material during these processes has received little attention.

We are studying the possible gene flow from oilseed rape (*Brassica napus*) to weedy *Brassica campestris*. We have previously shown that interspecific hybridisation between the two species occurs spontaneously under field conditions (Jørgensen and Andersen 1994). Hybrids are vigourous, resembling the *B. napus* parent, and have a variable fertility. Backcross plants (hybrid×*B. campestris*) have also been obtained under field conditions (Jørgensen et al. 1995).

B. napus is an amphidiploid (AACC, 2n=38) and *B. campestris* (AA, 2n=20) is considered to be one of the diploid progenitor species of *B. napus*, the other being *B. oleracea* (CC, 2n=18) (U 1935). Interspecific hybrids between *B. napus* and *B. campestris* have the genomic constitution AAC (2n=3x=29). Plants in the first backcross

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generation with *B. campestris* are expected to carry ten pairs of A-chromosomes and a number of C-chromosomes varying between 0 and 9 (AA+0–9 C-chromosomes, 2n=20–29). Genetically, as well as from a risk assessment point of view, it is of interest to characterise the transfer of genes from the interspecific hybrid to the backcross generations. Of particular importance is the behaviour of genes on the C-chromosomes, since these chromosomes have no homologoues with which to pair during meiosis. Previous studies have indicated that the transmission of C-chromosomes from the interspecific hybrid to the first backcross generation (BC_1) and to the F_2 generation occurs at ratios that are variable between C-chromosomes, and often lower than 50% (Chen et al. 1990; McGrath and Quiros 1990). This suggests that different parts of the oilseed rape genome might introgress into the *B. campestris* gene pool at different rates.

In the present study we have crossed several different genotypes of the two species, with *B. napus* as female. Interspecific hybrids were backcrossed to *B. campestris*, with hybrids as females, with the aim of producing a backcross progeny suitable for genetic marker analysis. The inheritance of *B. napus*-specific randomly amplified polymorphic DNA (RAPD) markers to the first backcross generation was analysed in a selected backcross family.

Materials and methods

Plant materials and crossings

The following varieties, lines and populations were used in the crosses: *B. napus*, cv Drakkar, a line of transgenic Drakkar (male sterile, BASTA resistant, serial no. 93B0144; Plant Genetic Systems, Belgium), a line of transgenic Westar (Round-up resistant, Line no. 111252; Monsanto Company, Missouri, USA), transgenic lines from a variety cross cv Karat×cv Loras (Round-up resistant, lines no. 9110090, 9110092, and 9110096; Danisco Seed, Holeby, Denmark); *B. campestris*, cvs Indus and Tobin, and individuals from weedy Danish populations. Furthermore, *Brassica oleracea* cultivars Erfu, Long Island and Hammenhøgs no. 32 were used for RAPD analysis.

Seeds were germinated on filter paper in Petri dishes and plants were transferred to pots at the cotelydon stage and grown in a growth chamber. *B. campestris* seeds were subjected to the following dormancy breaking treatment: 8 h/30 °C, 16 h/20 °C, three cycles, in continous dark (Toole and Toole 1939). Buds on female parents were emasculated two days prior to pollination and covered with a parchment bag. Pollen was applied to the buds and the pollinated buds remained covered for 7–10 days, until pods had developed. Seeds were harvested at maturity. Electrophoresis of phosphoglucomutase (PGM), performed according to Nielsen and Johansen (1986), was used to verify that randomly chosen offspring from controlled interspecific crosses, and all plants used for backcrossing, were indeed interspecific hybrids. All 51 plants analysed proved to be hybrids. The pollen fertility of all *B. campestris* and hybrid plants used for crosses was estimated by staining with cotton-blue (Phillips 1981). Four hundred pollen grains from two flowers were counted from each plant. Pollen fertility of 19 randomly chosen backcross plants was also estimated.

DNA extraction and RAPD analysis

DNA was extracted from leaves by the method of Edwards et al. (1991), with the following modifications: one phenol extraction and one chloroform:isoamylalcohol (24:1) extraction were made before precipitation with isopropanol. The precipitated DNA was dissolved in 100 µl of TE-buffer. Replicate, independent DNA extractions were made from two different leaves on each plant.

Twenty microliter RAPD reactions were run in microtiter plates essentially according to Williams et al. (1991) except for the following: 1.5 microliter of the DNA solution prepared as described above was used for each reaction, and the temperature cycle used was: 93 °C/5 min (one cycle); 93 °C/1 min, 36 °C/1 min, 72 °C/2 min (45 cycles); 72 °C/10 min (one cycle); 30 °C/1 sec (one cycle). Reactions were carried out on a Hybaid Omnigene temperature cycler with Super*Taq* polymerase (HT Biotechnologies, Cambridge, UK). Amplification products were analysed by agarose-gel electrophoresis (Sambrook et al. 1989), using 1.4% agarose gels. A total of 43 ten-base oligonucleotide primers from Operon Technologies, Inc. (Alameda, Calif., USA) were tried (B5, C14, C16, G1–G20, and H1–H20). Of these, 19 primers amplified 33 RAPD markers which proved suitable in the analysis of the backcross progeny BC151 [(*B. napus* cv Drakkar×*B. campestris* cv Indus)×*B. campestris* cv Indus], consisting of 92 plants. The markers were identified by their associated primer number and the approximate size of the amplified fragment in base pairs. All RAPD reactions were run on both preparations of DNA from each plant. In the few cases where inconsistency between the two replicates were found (most probably caused by factors such as DNA concentration or purity), reactions were run again or new DNA preparations were made.

Data analysis

Chi-square analysis was used to test for deviation from a 1:1 segregation ratio of individual markers. A significance level of 0.05 divided by 16 (the number of linkage groups plus the number of unlinked markers) was considered as appropriate. Thus, a value of 0.05/16=0.003 corresponds to a significance level of 5% for the occurrence of deviation from a 1:1 segregation. Since the number of independently segregating markers may actually be less than 16, the probability for a real deviation from a 1:1 segregation, judged by 0.003, may thus be less than 5%.

Chi-squares for independency of the observed segregation of pairs of markers were calculated and used to construct dendrograms by the furtherest, as well as nearest, neighbour methods (Pimentel 1979). As an additional test for co-segregation, pairs of markers with fewer than 5% recombinants among the backcross plants were considered to belong to the same linkage group. Based on these methods, groups of independently segregating markers were identified.

Results

Crosses

We first crossed different *B. napus* cultivars/transgenic lines with different *B. campestris* cultivars/populations, since parental genotype might influence the ease with which interspecific crosses and backcrosses could be made. All crosses were made with oilseed rape as the female parent, as this gives a higher seed set than the reciprocal cross (Downey et al. 1980). The pollen fertility of the *B. campestris* individuals used for crosses was higher than 87%. The results of these crosses are listed in Table 1. The average seed set was 9.8 seeds per pollination. Hybrids produced from some of these cross combinations, with a pollen fertility between 7% and 69% (average 43%, 18 plants analysed), were backcrossed to the parental *B. campestris* cultivar/population, with a resulting average seed set of 0.7 seed per pollination (Table 2).

Table 1 Interspecific crosses between *B. napus* and *B. campestris*

B. napus (female parent)	*B. campestris* (male parent)	Number of plants crossed (female/male)	Number of pollinations	Seeds/ pollination
Drakkar	Weedy	4/5	36	7.8
	Indus	1/1	12	20.0
	Tobin	1/1	11	12.8
Drakkar, transgenic line	Weedy	3/2	56	8.4
Westar, transgenic line	Weedy	2/2	24	11.6
	Indus	1/1	9	15.2
Karat × Loras, transgenic line 9110090	Weedy	2/2	44	11.2
	Indus	1/1	2	10.5
	Tobin	1/1	15	12.3
Karat × Loras, transgenic line 9110092	Weedy	3/3	31	9.9
	Indus	2/1	33	6.2
	Tobin	1/1	7	7.1
Karat × Loras, transgenic line 9110096	Weedy	4/4	71	9.2
	Indus	1/1	14	3.7
	Tobin	1/1	16	13.6
			Sum = 381	Average = 9.8

Table 2 Backcrosses between interspecific *B. napus* × *B. campestris* hybrids and *B. campestris*

Hybrid (female parent)	*B. campestris* (male parent)	Number of plants crossed (female/male)	Number of pollinations	Seeds/ pollination
Drakkar × weedy *B.c.*	Weedy	4/4	52	0.7
Drakkar, transgenic line × weedy *B.c.*	Weedy	5/3	206	0.3
Westar, transgenic line × weedy *B.c.*	Weedy	3/3	61	0.2
Karat × Loras, transgenic line 9110090 × weedy *B.c.*	Weedy	2/1	31	0.9
Karat × Loras, transgenic line 9110092 × weedy *B.c.*	Weedy	3/2	49	0.7
Karat × Loras, transgenic line 9110096 × weedy *B.c.*	Weedy	4/3	87	0.5
Drakkar × Indus	Indus	3/2	65	2.7
			Sum = 651	Average = 0.7

One type of backcross produced many more seeds than others, namely the cross (*B. napus* cv. Drakkar×*B. campestris* cv. Indus)×*B. campestris* cv. Indus. From this backcross, an average of 2.7 backcross seeds per pollination was obtained (Table 2). Ninety five seeds were produced on a single hybrid plant from 28 pollinations with the same male. Ninety two backcross plants grown from these seeds (backcross progeny BC151) were used for the analysis of transmission of RAPD markers. Pollen fertility was determined for 19 randomly selected plants from this progeny, and ranged between 11% and 84% (average 39%).

Genetic marker analysis

The parental *B. napus* and *B. campestris* individuals, as well as the hybrid plant and the *B. campestris* individual used in the backcross to produce the BC151 progeny, were all screened with 43 RAPD primers in order to identify RAPD markers specific to the parental *B. napus* individual. Suitable markers should be present in the *B. napus* parent and the hybrid (inherited from the *B. napus* parent) and absent in the two *B. campestris* male parents. This screening resulted in 33 suitable RAPD markers, amplified by 19 primers (Figs. 1, 2).

The 33 genetic markers were transmitted to the first backcross generation at frequencies ranging between 26% and 91% (Fig. 2). By testing each marker for deviation from a transmission rate of 50% - the rate expected if each marker corresponds to one locus with Mendelian segregation - it was found that only three markers deviated significantly: G2-600 (transmission frequency 77%), G13-590 (91%), and G14-325 (26%). The number of markers present in the backcross plants varied between two and 32.

Since we expected some of the markers to be inherited together in the backcross plants, e.g. because they are located on the same chromosome, we performed analyses of

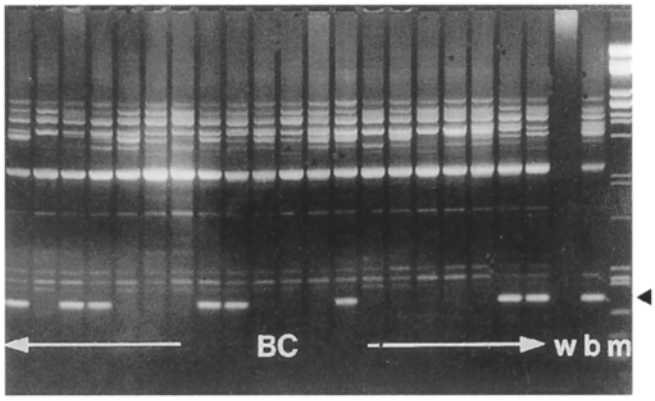

Fig. 1 Amplification of DNA from 20 plants from the backcross progeny BC151 with primer C16. The marker followed (C16-400) is indicated by an *arrowhead*. *BC* amplification of DNA from backcross plants; *w* amplification of water (negative control); *b* amplification of DNA from *Brassica napus* (positive control); *m* DNA molecular-weight marker (Lambda DNA cut with *PstI*)

A	*B*	*C*	*D*
G5-550 (55%)	B5-500 (37%)	G12-590 (39%)	B5-900 (51%)
G12-1425 (55%)	G6-1050 (37%)	G15-625 (39%)	G3-525 (51%)
G19-290 (55%)		H11-730 (42%)	H6-540 (51%)
H2-600 (55%)		H12-1300 (41%)	
H15-570 (55%)			

E	*F*	*Unlinked*
C16-400 (46%)	C14-900 (61%)	G2-380 (49%)
G3-205 (47%)	G9-275 (61%)	G2-600 (77%)*
G17-495 (45%)	G17-460 (60%)	G3-600 (48%)
G19-790 (45%)	H2-415 (60%)	G6-340 (55%)
	H2-1800 (61%)	G6-395 (39%)
		G9-460 (45%)
		G13-590 (91%)*
		G14-325 (26%)*
		H6-800 (46%)
		H11-1000 (62%)

Fig. 2 The 33 RAPD markers group into seven groups: six groups (*A-E*) with markers that are inherited together and a seventh (*unlinked*) consisting of markers that are not inherited together with other markers. The percentage given for each marker is the transmission frequency observed. * indicates markers with a transmission frequency significantly different from 50%

the pairwise segregation of markers by constructing dendrograms on the basis of nearest neighbour and furtherest neighbour analyses (Pimentel 1979). Because of the way the genetic markers were selected, we assumed that the majority of these markers are amplified only from the C-genome of *B. napus*. The large homology between the A-genomes in *B. napus* and *B. campestris* (Lydiate et al. 1993; Quiros et al. 1994; Teotonico and Osborn 1994) implies that most markers present in *B. napus* and absent in *B. campestris* are located on the C-genome of *B. napus*. This assumption was strengthened when we screened three additional individuals of *B. campestris* from a weedy Danish population and two individuals from each of three cultivars of *B. oleracea*. None of the 33 markers were found in any of the three *B. campestris* individuals, whereas 30 of the markers were present in at least one of the three *B. oleracea* cultivars. Three markers (G2-380, G3-600 and G14-325) were not found in *B. oleracea* (data not shown).

This indicates that the majority of the markers are indeed C-genome markers, and thus, we searched the data for pairs of markers where all, or almost all, of the backcross plants had either both or neither of the two markers. This is the segregation pattern expected for two markers amplified from the same C-chromosome, since the C-chromosomes have no homologues to recombine with. Barring illigitimate inter- and intra-genomic recombination and deletions, markers on the same C-chromosome are expected to co-segregate.

Dendrograms produced on the basis of nearest neighbour and furtherest neighbour analysis, and a search for co-segregation, tolerating a maximum of 5% plants with a recombinant genotype (i.e. one marker present, the other marker absent, or vice versa) (data not shown), gave identical results: 23 of the markers grouped in six groups, with between two and five markers per group. The remaining ten markers did not co-segregate with any of the other markers (Fig. 2).

A closer inspection of the segregation data for markers belonging to the same group, but where a few backcross plants with a recombinant genotype were found, gave interesting results. In group F, the five markers co-segregate in 91 of the 92 backcross plants and, in the single aberrant plant, both of the markers G17-460 and H2-415 are missing. Similarly, in group E, the four markers co-segregate in 88 out of the 92 plants. Among the four aberrant plants, two lack marker G17-495, one lacks marker G19-790, and one lacks both G19-790 and C16-400. These deviations from the co-segregation expected for markers present on the same C-chromosome, especially the observations where two markers are absent from the same plant, suggest that phenomena such as intra- or inter-genomic recombination or breakage of chromosomes took place.

Discussion

In all the interspecific cross combinations of the type *B. napus*×*B. campestris*, hybrid seeds were produced (Table 1). Thus, no strong influence of genotype on the crossability of the two species was observed. All of the backcross combinations also produced seed and, notably, a cross combination involving the *B. campestris* cultivar Indus produced more seed than other crosses, all made by using weedy Danish *B. campestris*.

Almost all of the 33 *B. napus*-specific RAPD markers were transmitted from the interspecific hybrid to the backcross progeny BC151 at frequencies that are not significantly different from 50%. Considering the two markers displaying a transmission frequency higher than 50%, one explanation could be that these markers are amplified from more than one locus in the genome. Thus, a marker ampli-

fied from two unlinked loci with equal segregation would have an expected transmission frequency of 75%. Therefore, the transmission frequency of marker G2-600 (77%) could result from amplification from two loci, and the transmission frequency of marker G13-590 (91%) could result from amplification from several loci. A transmission frequency of 26% (marker G14-325) is not easily explained.

The data analysis resulted in six groups of markers inherited together. Due to the way the markers were selected, we believe that these six groups of markers correspond to six of the nine C-chromosomes in *B. napus*. The transmission frequencies displayed by markers inherited together reflect the transmission frequency of the linkage group to which they belong. We can not entirely exclude the possibility that some markers are inherited together, even though they do not belong to the same chromosome. Such a phenomenon could occur, e.g., if plants carrying specific combinations of C-chromosomes were subject to selection.

The ten RAPD markers which did not co-segregate with other markers might be amplified from the three remaining C-chromosomes, or from the A-chromosomes of *B. napus*. The three markers with extreme transmission frequencies and the three markers not found in the screening of *B. oleracea* cultivars are all unlinked. It is tempting to speculate that the latter three markers might not be C-genome markers, but located on the A-genome of *B. napus*. The fact that they occur in the group of unlinked markers might simply be due to the fact that most markers are C-genome markers. Thus, only a very small chance exists that the few A-genome markers will be linked.

Most of the transmission frequencies observed in this study seem to be in accordance with the theory of equal segregation. McGrath and Quiros (1990) investigated the transmission rates of C-chromosomes in the same type of backcross as described in the present study, using diagnostic C-genome chromosome markers (RFLPs and isozymes). They found a mean C-chromosome transmission rate of 32.1% in one backcross family (27 individuals analysed), with transmission of single chromosomes ranging between 23.1 and 42.3%. Chen et al. (1990) investigated the loss of C-chromosomes during selfing of *B. napus*×*B. campestris* interspecific hybrids. Eight isozyme loci on C-chromosomes were transmitted to the F_2 progeny at extremely different frequencies (3–100%).

Analyses of the transmission of the extra chromosome in monosomic addition lines also shed light on the transfer of single, unpaired *Brassica* chromosomes. Chen et al. (1992) reported on a *B. campestris-alboglabra* (*oleracea*) monosomic addition line where, upon selfing, only 30.5% of the offspring contained the alien chromosome. This et al. (1990) dissected the *B. nigra* B-genome by making *Diplotaxis erucoides-B. nigra* addition lines. They selfed and backcrossed five monosomic addition lines with five different B-chromosomes, and found transmission frequencies of the alien chromosomes ranging from 0 to 30%. Other observations show that alien chromosomes are sometimes transferred at very high frequencies (see for example Chevre et al. 1991).

At present we are not able to explain why the transmission frequencies observed in this study seem more in accordance with a theory of equal segregation than those observed by others. Since the backcross progeny analysed was the only backcross combination out of seven that gave us sufficient offspring for genetic analysis (Table 2), it is tempting to speculate that the combination of *B. napus* cv Drakkar and *B. campestris* cv Indus somehow promotes and/or stabilises the transfer of C-chromosomes to backcross plants.

We present observations where a few individuals in the backcross progeny have only some of the markers of a linkage group. These observations suggest that intra- or intergenomic recombination or deletions are taking place. Both intra- and inter-genomic chromosome pairing have been described in *Brassica* species (Armstrong and Keller 1982; Attia et al. 1987). Intergenomic recombination between the A- and C-genomes has been reported by, e.g., McGrath et al. (1990) and Chen et al. (1992), and observations indicating intragenomic recombination in the C-genome have been reported by McGrath and Quiros (1990) and Quiros et al. (1994). Chromosome deletions of alien C-chromosomes in monosomic addition lines have been reported previously by, e.g., Hu and Quiros (1991). We are unable to distinguish between recombination and deletion in our data, but our results confirm the complex nature of the genetic processes occurring during hybridisation and backcrossing in the *Brassica* species.

Taken together, the results presented in this study on the genetics of introgression from oilseed rape to *B. campestris* indicate that genes present on the C-chromosomes can be transferred at comparatively high frequencies to the first backcross generation. As already discussed, other studies have indicated that some *B. napus* genetic markers are transferred at very low frequencies to backcross and F_2 generations with *B. campestris*. Chromosomal regions with a very low probablity of transfer in such interspecific crosses and backcrosses could hypothetically serve as targets for the "safe" integration of transgenes, via homologous recombination. Transgenes integrated in such a chromosomal region would, according to this hypothesis, have a reduced probability of transfer to the weedy species. In our study, all 33 markers analysed were transferred to the backcross plants, and the lowest transfer frequency observed was 26%. Thus, the hypothesis of possible "safe" integration sites is not supported by our results.

The observations (1) that oilseed rape and *B. campestris* seem to hybridise readily under field conditions (Jørgensen and Andersen 1994), (2) that interspecific hybrids can backcross with *B. campestris* under field conditions (Jørgensen et al. 1995), and (3) that oilseed rape genetic markers can be transferred at relatively high frequencies to the first backcross generation (this manuscript), all indicate that flow of transgenes from transgenic oilseed rape to *B. campestris* may occur.

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