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ORIGINAL PAPER

Genotypic effects on the frequency of homoeologous and homologous recombination in *Brassica napus* × *B. carinata* hybrids

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Abstract We investigated the influence of genotype on homoeologous and homologous recombination frequency in eight different *Brassica napus* (AAC^nC^n) × *B. carinata* (BBC^cC^c) interspecific hybrids (genome composition C^nC^cAB). Meiotic recombination events were assessed through microsatellite marker analysis of 67 unreduced microspore-derived progeny. Thirty-four microsatellite markers amplified 83 A-, B-, C^n- and C^c-genome alleles at 64 loci, of which a subset of seven markers amplifying 26 alleles could be used to determine allele copy number. Hybrid genotypes varied significantly in loss of A- and B-genome alleles ($P < 0.0001$), which ranged from 6 to 22% between hybrid progeny sets. Allele copy number analysis revealed 19 A–C, 3 A–B and 10 B–C duplication/deletion events attributed to homoeologous recombination. Additionally, 55 deletions and 19 duplications without an accompanying dosage change in homoeologous alleles were detected. Hybrid progeny sets varied in observed frequencies of loss, gain and exchange of alleles across the A and B genomes as well as in the diploid C genome. Self-fertility in hybrid progeny decreased as the loss of B-genome loci (but not A-genome loci) increased. Hybrid genotypes with high levels of homologous and homoeologous exchange may be exploited for genetic introgressions between *B. carinata* and *B. napus* (canola), and those with low levels may be used to develop stable synthetic *Brassica* allopolyploids.

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Introduction

The *Brassica* "U's triangle" consists of three diploid species, *Brassica rapa*, *B. nigra* and *B. oleracea*, with diploid genome complements AA = 20, BB = 16 and CC = 18, respectively, and three allotetraploid species *B. juncea*, *B. napus* and *B. carinata*, with genome complements AABB = 36, AACC = 38 and BBCC = 34, respectively (U 1935). Each of the allotetraploid species are derived from hybrids between the diploid species (Mizushima 1980). The diploid species were of polyploid origin with a common hexaploid ancestor (Lysak et al. 2005). The A and C genome diverged more recently from a common ancestor than from the more distant B genome (Warwick and Black 1991).

Interspecific hybrids between each of the six "U's triangle" species have previously been created, either to introduce valuable alleles from wild relatives into crop species through genome introgression (Choudhary et al. 2000; Roy 1984) or for the purposes of elucidating the relationship between the A, B and C genomes through meiotic pairing analysis (reviewed by Mizushima 1980). In describing the genome structure of *Brassica* species, the genomes are often distinguished by their origin in diploid or allotetraploid species. For example, the A subgenome from *B. rapa* was designated as A^r, whereas that from *B. napus* was designated as A^n (Li et al. 2004). Analyses of homoeologous and homologous pairing behaviour in these interspecific hybrids are useful in predicting the probability

of genome introgression, as well as the potential stability of newly created allopolyploid hybrids.

Unreduced gametes (pollen and ovules with the somatic 2*n* chromosome number) have been observed in a number of *Brassica* species at low frequencies (Eenink 1975; Fukushima 1930; Heyn 1977; Mackay and Low 1975; Moyes et al. 2002; Sheidai et al. 2006; Tokumasu 1965), and tend to be produced at a higher frequency in interspecific hybrids than in the parental species (Ramsey and Schemske 1998). Recently, it was reported that an interspecific hybrid (C^nC^cAB) between *B. napus* (AAC^nC^n) × *B. carinata* (BBC^cC^c) produced a high proportion (26/28) of microspore-derived progeny with approximately the same chromosome complement ($C^nC^cAB = 4x = 36$) as the hybrid parent, and that unreduced gametes by first division restitution were the source of these progeny (Nelson et al. 2009). First division restitution occurs when homologous, non-sister chromatids fail to segregate into separate gametes at meiosis I.

Molecular marker analysis of unreduced gamete-derived progeny of C^nC^cAB hybrids permits inferences to be made regarding homologous and homoeologous recombination events during meiosis. Techniques such as dosage-sensitive molecular markers and fluorescent in situ hybridisation provide reliable estimates of homoeologous recombination frequencies (Gaeta and Pires 2010). Microsatellite and other molecular markers can be used to estimate the number of copies of an allele present in the genome, often referred to as "allele copy number analysis". This technique has been used to detect abnormal meiotic recombination events through progeny analysis in both *Arabidopsis* (Henry et al. 2006) and *Brassica* polyploids (Nicolas et al. 2007; Szadkowski et al. 2010).

In unreduced gamete-derived progeny of C^nC^cAB hybrids, homologous recombination events resulted in reduced C-genome heterozygosity at loci genetically distant from centromeres (Nelson et al. 2009). Homoeologous recombination can cause a loss of microsatellite loci in the A and B genomes, through homoeologous non-reciprocal translocations (HNRTs). Further support for the occurrence of HNRTs between several pairs of A/C and B/C chromosomes in this system was provided by a proportion of the markers which detected simultaneous duplication/deletion of loci through copy number analysis (Nelson et al. 2009). Homoeologous chromosome pairing during metaphase I in *B. napus* × *B. carinata* hybrids was observed by Mason et al. (2010) using fluorescent in situ hybridisation, which supported the role of homoeologous recombination in generating abnormal meiotic products in this hybrid type.

We have expanded the analysis of Nelson et al. (2009) to a population of 80 progeny derived from microspore culture from eight different *B. napus* × *B. carinata* hybrids (involving five varieties of *B. napus* and two varieties of *B. carinata*). We tested the hypothesis that the level of homoeologous and homologous pairing during meiosis in the interspecific hybrids would be affected by hybrid genotype. Microsatellite markers were used for dosage-sensitive marker analysis of allele copy numbers in the unreduced microspore-derived progeny, supplemented by presence/absence results for additional loci.

Materials and methods

Generation and growth of microspore-derived progeny

The parent varieties were *B. napus* cultivars 'Surpass 501TT', 'Trilogy', 'Tristate', 'Trigold' and 'Surpass400-024DH' (a selection from 'Surpass 400' derived by microspore culture) were supplied by Canola Breeders Western Australia Pty Ltd, Perth, Australia, and *B. carinata* lines '193467.7.1' and '94024.2' were selections made by Margaret Campbell of The University of Western Australia from accessions ATFCC 193467 and ATFCC 94024 from the Australian Temperate Field Crops Centre, Horsham, Australia. *B. napus* 'Trilogy' and 'Tristate' were doubled haploid varieties with very low levels of heterozygosity, whereas *B. napus* 'Trigold' and 'Surpass 501TT' and *B. carinata* '193467.7.1' and '94024.2' were open-pollinated varieties with various levels of heterozygosity.

The five genotypes of *B. napus* (maternal parent) were crossed with both genotypes of *B. carinata*, and isolated microspore culture (as described by Nelson et al. 2009) was performed on hybrid plants from ten interspecific combinations. Three separate microspore isolations were carried out from anthers of each hybrid plant. Microspore-derived progeny were grown in a controlled environment room at 18°C/10°C day/night. Cuttings of each line were subjected to 0.5% colchicine treatment for 0.5 h followed by washing for 2 h. Cuttings were then rooted and grown to maturity over summer in Perth, Western Australia, in a glasshouse with evaporative cooling. Self-pollination was enforced by enclosing racemes in pollination bags. Self-fertility was defined as the ability to produce at least one seed inside selfing bags.

Molecular markers

Microsatellite (simple sequence repeat, SSR) markers spanning the A and C genomes of *B. napus* and the B genome of *B. juncea* were provided by A. Sharpe and D. Lydiate (Agriculture and AgriFood Canada Saskatoon Research Centre, Saskatoon; personal communication; for more information, see http://brassica.agr.gc.ca). Microsatellite markers were used to assess presence or absence of chromosome segments in microspore-derived progeny compared with the interspecific hybrids and *B. napus* and

B. carinata parents and for copy number effects as described in Nelson et al. (2009). Microsatellite marker fragment analysis was performed using an AB3730*xl* DNA sequencer (Applied Biosystems, Scoresby, Victoria) and agarose gel electrophoresis as described in Nelson et al. (2009).

Across all hybrid progeny sets, 34 microsatellite primer pairs identified 64 loci and 83 alleles in the A, B and C genomes, and an additional 9 A–C and 19 B primers amplified 36 more alleles at 32 additional loci in progeny set H1-2. Progeny set H1-2 was the subject of a previous investigation (Nelson et al. 2009). Microsatellite markers were chosen to provide a more-or-less even coverage of the genomes: two to six loci per chromosome for the A and B genomes and two to four polymorphic loci per chromosome for the C genome. For each individual, 27–36 A-genome loci and 16–35 B-genome loci were scored for presence or absence of alleles, and 19–23 C-genome loci were scored for segregation of alleles from *B. napus* (C^n) and *B. carinata* (C^c).

Seven of these microsatellite primer pairs (amplifying 26 alleles at 19 loci on the A, B, and C genomes) gave unambiguous dosage intensity results, and were selected for copy number analysis. Five of these markers detected loci on A, B and C genomes (each marker potentially amplified four alleles: A, B, C^n and C^c), and two markers detected loci on A and C only (each potentially amplifying three alleles: A, C^n and C^c). Each unreduced gamete-derived individual in the population was scored for these 7 microsatellite markers, to give a maximum of 325 unambiguous marker × individual observations. Each of these observations was assigned to one of four categories based on the presence, absence and relative intensity of alleles from each genome: (i) loss of one allele (absence of one allele from A, B, C^n or C^c) with no other changes recorded; (ii) gain of one allele (doubling of band intensity of either A, B, C^n or C^c allele) with no other changes visible; (iii) both gain and loss of alleles in different genomes amplified by same microsatellite marker (absence of one allele from either A, B, C^n or C^c, with a clear doubling of band intensity in one of the remaining alleles); and (iv) no change in presence of alleles from expected for unreduced gamete-derived progeny (all expected alleles present at expected band intensity, allowing for segregation at heterozygous C-loci). A few observations which could not be unequivocally placed in categories (i), (ii), (iii) or (iv) were also recorded. The data were assessed for differences among hybrid progeny groups for the number of observations in each category.

Statistical analyses

Statistical analyses were carried out using statistical packages R 2.11.1 (R Development Core Team, Vienna, Austria, 2008) and Genstat Version 9.1.147 (VSN International Ltd). Linear mixed models followed by Tukey's HSD were used to establish overall differences between progeny sets in presence of A-, B- and C-genome loci. REML variance components analysis was used to determine the effect of loss of loci on fertility. Fisher's exact test for count data (with Monte-Carlo simulation: 1,000,000 replicates for large samples) was used to establish differences between hybrid progeny sets for observations of loss, duplication and exchange of alleles as detected through copy number analysis.

Results

Microsatellite marker characterisation of the population derived from microspores of C^nC^cAB hybrids

A total of 80 progeny derived from microspore culture of 10 different C^nC^cAB (*B. napus* × *B. carinata*) hybrids survived to flowering (Table 1), including the 28 progeny from CCAB hybrid H1-2 which were analysed by Nelson et al. (2009). Microsatellite markers revealed that 67 of these progeny were heterozygous for C^n and C^c alleles at 41–89% of the loci tested, consistent with their formation from unreduced microspores in which homologous C genome, non-sister chromatids failed to segregate (i.e. a process of first division restitution). On average for these progeny, 6% of A- and B-genome linkage groups were lost based on the two or more loci examined per linkage group, and 10% of A- and B-genome linkage groups showed at least one present and one absent locus. For the remaining 13 progeny, C-genome loci appeared to be segregating normally: C-genome loci were heterozygous at 0–16% of loci tested, consistent with formation from reduced gametes. On average, 24% of A and B genome chromosomes were missing in these 13 individuals (two to seven), with one to four detectable homoeologous recombination events in the A and B genomes per individual. None of these 13 putative reduced gamete-derived individuals set self-seed. Five maternal parents and two paternal parents were represented in the provenance of the 67 unreduced gamete-derived progeny (Table 1). The proportion of unreduced microspore-derived progeny produced was not significantly affected by hybrid genotype ($P = 0.16$, Fisher's exact test for count data).

Within some hybrid progeny sets, two or more progeny were observed with the same pattern of marker loci across each chromosome in the A, B and C genomes, i.e. identical haplotypes. If identical haplotypes were present in the same microspore isolation group, then the duplicated haplotypes were removed from further analysis (Table 1). On two occasions, identical haplotypes were present in different microspore isolations from the same progeny set, and these were retained in the analysis. Identical haplotypes were

Table 1 Production of unreduced (U) and reduced (R) microspore-derived progeny from ten interspecific (*B. napus* × *B. carinata*) hybrids

B. napus parent (♀)	*B. carinata* parent (♂)	Interspecific hybrid progeny set[a]	No. R progeny	No. U progeny[b]
′Trilogy′	′94024.2′	H1-1	2	6 (5)
′Trilogy′	′94024.2′	H1-2	2	26 (18)
′Trilogy′	′94024.2′	H1-3	0	2 (1)
′Trilogy′	′94024.2′	H1-4	0	2 (2)
′Trilogy′	′193467.7.1′	H2	0	8 (8)
′Tristate′	′94024.2′	H3	5	7 (7)
′Trigold′	′94024.2′	H4	0	3 (3)
′Surpass400_024DH′	′193467.7.1′	H5-1	4	9 (9)
′Surpass400_024DH′	′193467.7.1′	H5-2	0	1 (1)
′Surpass 501TT′	′94024.2′	H6	0	3 (3)
		Total	13	67 (57)

The U and R status of microspore-derived progeny was determined by microsatellite markers showing segregation of *B. napus* and *B. carinata* C-genome alleles. Two discrete distributions of progeny were observed. Progeny in which 41–89% of C-genome loci showed presence of both C-genome alleles were assumed to be U progeny, and progeny in which 0–16% of C-genome loci showed presence of both C-genome alleles were assumed to be R progeny

[a] The hybrid code for each parental combination (e.g. H1) is followed by the hybrid progeny set number. For example, hybrid progeny sets H1-1, H1-2 and H1-3 were from different hybrid plants, but from the same parental combination

[b] The number in parentheses is the number of unique haplotypes after removing duplicated haplotypes from the same microspore isolation. Whenever two or more progeny were observed with identical haplotypes in the same microspore isolation, then the duplicate haplotypes were removed from further analysis

observed in three progeny sets: H1-1 and H1-3 each had one pair of identical haplotypes; progeny set H1-2 contained two groups of five and a group of three identical haplotypes (Table 1). Two progeny sets (H1-3 and H5-2) contained only a single unique individual (Table 1), and hence were excluded from statistical analysis of genotypic differences, although otherwise retained. Hence, a total of 55 unreduced gamete-derived progeny with distinctive molecular marker results from eight hybrid parents were used in subsequent analysis of homologous and homoeologous recombination.

Allele presence and absence across the A, B, C^n and C^c genomes in the experimental population

Allele transmission from the *B. napus* × *B. carinata* hybrid parents into the unreduced gamete-derived progeny ranged from 73 to 100% in the A genome and 63 to 100% in the B genome (Table 2). Allele transmission in the C^n genome ranged from 67 to 97%, and allele transmission in the C^c genome ranged from 67 to 100% (Table 2). No linkage group retained all loci across the whole experimental population.

Genetic effects on transmission of A- and B-genome alleles from the hybrid parent into the unreduced microspore-derived progeny

Total loss of A- and B-genome alleles ranged from 6 to 22% between different progeny sets in the experimental population and was linearly modelled using paternal parent (*B. carinata*) genotype, maternal parent (*B. napus*) genotype and interspecific hybrid parent as explanatory variables in the same model. Neither paternal parent genotype nor maternal parent genotype had a significant effect on loss of A-, B- or combined A- and B-genome loci (Table 3). Hybrid parent showed a significant effect on loss of loci in the A, B and combined A and B genomes (Table 3).

Differences between genotypes in frequency of recombination events involving the A- and B-genome linkage groups

For linkage groups in the A and B genomes where at least two loci were amplified in each of the eight progeny sets, observations of these linkage groups were classified into "present"—no missing loci and "absent"—all missing loci. On chromosomes which showed one or more present and one or more absent loci, one or two recombination events were detectable per linkage group from the pattern of missing loci (e.g. 1-0-0 was assumed to indicate one recombination event, and 1-0-1 to indicate that two recombination events had occurred). Progeny sets varied in frequency of recombination events detected in this way (Table 4). The numbers of present and absent linkage groups were not significantly different between progeny sets (Table 4), but progeny sets varied in the distribution of observations between present, absent and recombination categories (Table 4).

Table 2 % Transmission of *Brassica* A-,B- and C-genome alleles in a population derived from unreduced microspores of *B. napus* × *B. carinata* interspecific hybrids

Chromosome	A1	A1	A1	A2	A2	A2	A3	A3	A3	A3	A3	A4	A4	A4	A4*	A5	A5	A5	A6	A6	A7	A7	A7	A7	A8	A8	A8	A9	A9	A9	A9	A9	A10	A10*	A10	A10
Approx. cM	0	10.9	59.8	20.1	43.4	97.5	3.3	45.9	68.5	94.4	108.9	10.2	20.3	33.6	?	0	48.3	106.7	3.5	64.5	0	58.1	75.1	101.9	6.7	6.7	27	0	7.9	37.4	51	105.5	0	?	33.6	68.5
Marker	sN11641 FAM	sN11707 FAM	sN3523R NED	sR94102 VIC	sR6293	sNRE30 NED	sR12137I FAM	sR12015 VIC	sN11722 FAM	sR11644	sN1919 FAM	sN13034 FAM	sN2025	sN11516 PET	sN2552 VIC	sNRD03 FAM	sN12353 NED	sR9555 PET	sN0539	sS1949 NED	sR0282R	sR7223	sR12387	sNRA59	sR7178 PET	sR9480	sR3688	sR9447	sN1988 VIC	sR9251J	sR12777	sR5795	sN13039 FAM	sORH13 NED	sS2066 PET	sN8502
Alleles present	31	39	38	46	48	47	46	40	19	44	17	42	42	14	36	47	49	38	39	47	46	19	18	46	51	18	49	39	48	48	47	52	46	30	50	48
No. observations	38	45	47	54	51	55	53	50	20	46	18	51	54	19	44	51	54	43	51	54	51	20	18	48	54	18	51	44	52	52	50	55	47	33	55	52
% transmission	79%	73%	81%	84%	94%	86%	87%	80%	95%	96%	94%	83%	79%	74%	82%	91%	89%	86%	77%	88%	90%	95%	100%	96%	95%	100%	96%	89%	93%	93%	94%	95%	98%	91%	91%	92%

Chromosome	B1*	B1	B1	B1	B2	B2	B2	B2	B2	B3	B3	B3	B3	B4	B4	B4	B4	B4	B5	B5	B5	B5*	B5	B6	B6	B6	B6	B6	B7	B7	B7	B7	B8	B8	B8
Approx. cM	?	0	34.5	78.3	0	10	45.7	59.1	65.8	17.5	37.7	41.1	51.1	0	16.8	20.1	26.8	40.4	0	6.6	9.9	?	19.9	35.5	45.6	59.2	82.9	104.1	0	25.8	29.5	64.4	24.8	24.8	52
Marker	sR9555 PET	sJ3838F	sJ84165	sB05631	sJ0846	sJ2338RI	sJ103104	sJ7079	sB48178	sB1822	sB1672	sJ7046	sB1990F	sB2131	sA0306	sB0372	sB2141AI	sB1935A	sB0202I	sB3140	sJ38744	sN11722 FAM	sJ6842	sJ7104	sJ0338	sJ1505	sJ3640I	sJ0502	sB1871	sJ39119I	sJ1536	sJ4633	sB1728	sJ16681	sB3751
Present	50	46	18	44	53	18	18	18	48	51	18	18	54	48	17	17	17	17	17	51	17	17	50	50	18	18	51	18	48	15	17	52	35	13	45
No. observations	54	52	18	54	54	18	18	18	53	53	18	18	55	50	18	18	18	18	18	54	18	19	55	53	18	18	54	18	54	16	17	55	55	17	55
% transmission	93%	89%	100%	82%	98%	100%	100%	100%	91%	96%	100%	100%	98%	96%	94%	94%	94%	94%	94%	95%	94%	89%	91%	95%	100%	100%	95%	100%	87%	94%	100%	93%	63%	76%	80%

Chromosome	C1	C1	C1	C2	C2	C3	C3	C3	C4	C4	C4	C5	C5	C6	C6	C6	C7	C7	C7	C8	C8	C8	C9	C9	C9
Approx. cM	10.9	10.9	91.9	26.3	77	69.2	100.2	133.7	23.6	74.7	106.2	10.1	95.4	29.3	85.3	116.6	38.4	75.8	124.5	13.4	16.8	122.9	20.8	54.3	67.7
Marker	sN11641 FAM	sN11707 FAM	sN3523R NED	sR94102 VIC	sNRE30 NED	sR12015 VIC	sNRA56	sN2032	sN12353 NED	sN11516 PET	sN2552 VIC	sORH13 NED	sR9555 PET	sS2486 FAM	sN2834 PET	sR12387	sNRH63	sS1949 NED	sN1919 FAM	sR7178	sS2331B	sORB29A FAM	sN1988 VIC	sR9251J	sS2066 PET
Present	38	37	36	40	46	38	48	36	46	15	50	49	35	50	43	15	48	39	12	15	42	52	49	46	48
No. observations	45	46	46	48	55	49	51	37	54	20	55	55	47	53	55	17	54	46	18	18	50	55	55	51	54
% transmission C^n	85%	79%	77%	84%	84%	78%	94%	97%	86%	75%	91%	89%	76%	95%	79%	88%	88%	73%	67%	83%	84%	95%	86%	91%	88%
Present	42	52	38	42	47	42	-	-	36	13	43	49	45	42	43	14	51	48	15	18	49	42	49	50	44
No. observations	46	54	47	54	55	49	-	-	53	19	55	55	54	53	55	18	54	54	18	18	52	55	55	52	55
% transmission C^c	90%	96%	81%	77%	86%	86%	-	-	67%	68%	79%	88%	82%	80%	79%	78%	95%	89%	83%	100%	92%	77%	89%	96%	79%

Markers with 18 or fewer observations were tested on one progeny set (H1-2 only). Not all progeny sets amplified all alleles

[a] Location inferred

Table 3 Average proportion of microsatellite marker loci missing from the A genome (P_A), from the B genome (P_B) or from both A and B genome (P_{A+B}) in 55 unreduced microspore-derived progeny of *B. napus* × *B. carinata* hybrids

No. plants	Paternal parent	Maternal parent	Interspecific hybrid	P_{A+B}	P_A	P_B
5	′94024.2′	′Trilogy′	Hl-1	0.16^{ab}	0.19^{ab}	0.12^{ab}
18	′94024.2′	′Trilogy′	Hl-2	0.06^{d}	0.07^{b}	0.05^{b}
2	′94024.2′	′Trilogy′	Hl-4	0.12^{abcd}	0.16^{ab}	0.03^{ab}
8	′193467.7.1′	′Trilogy′	H2	0.10^{bcd}	0.13^{ab}	0.05^{b}
7	′94024.2′	′Tristate′	H3	0.17^{ac}	0.16^{ab}	0.19^{a}
3	′94024.2′	′Trigold′	H4	0.22^{a}	0.22^{a}	0.24^{a}
3	′94024.2′	′Surpass 501-TT′	H5-1	0.18^{ab}	0.20^{ab}	0.15^{ab}
9	′193467.7.1′	′Surpass400-024DH′	H6	0.08^{bd}	0.06^{b}	0.10^{ab}

Data are given as group averages. Maternal, paternal and interspecific hybrid effects were analysed using linear models followed by Tukey's HSD test. Maternal and paternal effects were not significant using the model: proportion of loci ~ interspecific hybrid + maternal parent + paternal parent. The effect of hybrid parent on P_{A+B}, P_A and P_B was highly significant ($P < 0.00001$, $P = 0.0016$ and $P = 0.0010$, respectively). Numbers followed by the same superscript letter in columns are not significantly different at $P = 0.05$

Detection of allele duplication and deletion events in the *Brassica* A, B, C^n and C^c genomes through microsatellite marker allele copy number analysis

Different categories of non-homologous recombination between genomes were identified in the unreduced gamete-derived population for the 325 unambiguous marker × individual observations (Table 5). These exchanges were inferred by loss (deletion) of a microsatellite marker locus in one genome, gain of a locus (doubling of signal intensity) in one genome, and loss coupled with the simultaneous gain (duplication) of the homoeologous locus in

Table 4 Transmission of *Brassica* A- and B-genome linkage groups in eight different progeny sets derived from unreduced microspores of *B. napus* × *B. carinata* hybrids

	Hl-1	Hl-2	Hl-4	H2	H3	H4	H5-1	H6	Significance[a]
Present	60 (76%)	262 (92%)	27 (84%)	110 (88%)	78 (72%)	32 (74%)	112 (82%)	32 (73%)	$P = 0.89$
Absent	7 (9%)	14 (5%)	0 (0%)	3 (2%)	10 (9%)	7 (16%)	3 (2%)	5 (11%)	$P = 0.16$
Recombinations	12 (15%)	10 (3%)	5 (16%)	12 (10%)	21 (19%)	4 (9%)	22 (16%)	7 (16%)	$P = 0.011$

Linkage groups with two or more loci observable across all progeny sets were scored as "Present" if all loci were present and "Absent" if all loci were absent on that linkage group. "Recombination" was scored as one event or two events per linkage group depending on the pattern of lost and retained loci (e.g. 1-0-0 for sequential loci as one recombination event, 0-1-0 for sequential loci as two recombination events). Data are given as the number of observations in each category followed by the % from the number of observations of each event (out of the total number of observations (given by the sum of the linkage groups observed across all individuals in a progeny set)

[a] Fisher's exact test for count data, comparing observations of each genotype against the population average (expected values corrected for total number of observations in each progeny set). The overall relationship between categories and genotypes was significant at $P < 0.0001$ (Fisher's exact test for count, data with Monte-Carlo simulation.)

Table 5 Categories of allele inheritance in unreduced microspore-derived progeny (genome complement C^nC^cAB) in eight hybrid progeny sets derived from *B. napus* (AAC^nC^n) × *B. carinata* (BBC^cC^c) parents, as revealed by copy number analysis

Hybrid progeny set[a]	Category (i): loss of one allele	Category (ii): gain of one allele	Category (iii): gain and loss of allele	Category (iv): "normal" unreduced gamete	Total
H1-1	9	2	4	15	30
H1-2	8	10	7	65	90
H1-4	2	1	2	9	14
H2	3	4	7	39	53
H3	12	1	4	24	41
H4	5	0	2	15	22
H5-1	13	1	3	42	59
H6	3	0	3	10	16
Total (%)	55 (16.9%)	19 (5.8%)	32 (9.8%)	219 (67.4%)	325

Seven markers amplifying 26 alleles in the A, B and C genomes across 55 unique individuals were used for copy number analysis. Unambiguous observations for each set of alleles amplified by a single marker (A, B, C^n and C^c for 5 markers and A, C^n and C^c for 2 markers) were sorted into four categories, which are further explained in "Materials and methods"

[a] Hybrid progeny sets varied in distribution of observations between categories ($P = 0.03$, Fisher's exact test for count data with Monte-Carlo simulation)

another genome (Table 5). Variation was observed between hybrid progeny sets in the patterns of duplications and deletions of alleles (Table 5). One of these microsatellite markers (sN1988) was selected to demonstrate the assignment of observations to categories, based on duplication or absence of signal intensity for individuals in all hybrid progeny groups (Fig. 1). Markers varied in their patterns of allele duplication, deletion and exchange ($P < 0.0001$, Fisher's exact test for count data with Monte-Carlo simulation, Supplementary Table 1).

A number of inferences were made regarding homoeologous exchanges from a more detailed examination of the marker × individual observations on the 55 unique unreduced microspore-derived individuals (Supplementary Table 2). There were 11 homoeologous reciprocal exchanges between the A genome and the *B. napus* C genome (C^n), 8 exchanges between the A genome and the *B. carinata* C (C^c) genome, 7 exchanges between the B genome and C^n genome, 3 between the B genome and the C^c genome and 3 between the A and B genomes. There were 55 deletions of a locus without a corresponding duplication at a homoeologous locus, and this occurred at similar frequencies in each of the A (19), B (15), C^n (11) and C^c (10) genomes (Supplementary Table 2). Finally, 19 duplications of an allele occurred without a corresponding deletion in the A (7), B (3), C^n (4) and C^c (5) genomes (Supplementary Table 2).

Genetic effects on homologous and non-homologous C genome interactions revealed by microsatellite marker allele copy number analysis

Microsatellite allele copy number analysis revealed that hybrid progeny sets had significantly different patterns of allele inheritance in the C genome (Table 6). Progeny sets also had significantly different patterns of allele inheritance

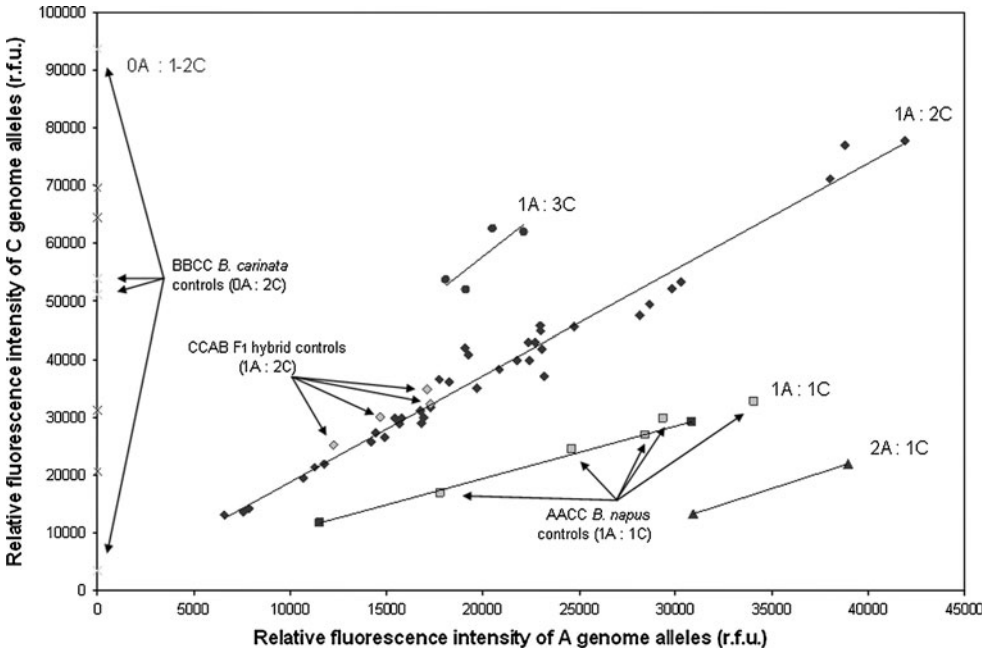

Fig. 1 Number of copies of *Brassica* A and C genome alleles amplified by microsatellite marker sN1988 in individuals derived from unreduced microspores of *B. napus* (AAC^nC^n) × *B. carinata* (BBC^cC^c) hybrids (genome composition C^nC^cAB). In the absence of homoeologous exchange or other meiotic events resulting in chromosome or chromosome fragment loss, individuals in this experimental population are expected to have two C genome alleles [one each from *B. napus* (C^n) and *B. carinata* (C^c)] and one A genome allele (from *B. napus*), since they are derived from unreduced gametes in which meiosis failed to segregate homologous chromatids. The signal intensities of the marker alleles were measured on an AB 3730*xl* capillary DNA sequencer. Relative intensity of A-genome alleles (*x*-axis) comparative to C-genome alleles (*y*-axis) is plotted in relative fluorescence units (r.f.u.). Five distinct distributions were distinguished (as follows), with parent and hybrid controls (indicated on figure) providing support for copy number allocations: *0A:1C* or *0A:2C* loss of an A genome allele through either a recombination event with another chromosomal region not covered by this marker (paralogous recombination); or loss of an A genome chromosome or chromosome fragment through chromatid splitting, nuclear exclusion or other cytological abnormalities at meiosis I. *1A:3C* gain of a C genome allele through either homoeologous non-reciprocal exchange with a homoeologous region not covered by this marker, failed C genome chromatid separation at meiosis II, or an unequal homologous exchange. *1A:2C* no homoeologous non-reciprocal exchange or loss of alleles (same complement as parent C^nC^cAB F_1 hybrid, expected under "normal" non-reduction model for meiosis). *1A:1C* loss of a C genome allele through either a recombination event with another chromosomal region not covered by this marker (paralogous recombination); formation of a heterozygote inversion loop between *B. napus* and *B. carinata* C genome chromosomes or unequal crossing over. *2A:1C* homoeologous non-reciprocal exchange between A and C genome alleles in the homoeologous regions covered by this marker

solely as a result of homologous recombination events converting $1C^n$:$1C^c$ to $2C^n$:$0C^c$ and $0C^n$:$2C^c$ ($P = 0.008$, Fisher's exact test for count data with Monte-Carlo simulation, Table 6). Non-homologous recombination events involving the C^n genome occurred at the same frequency as events involving the C^c genome (total frequency of 21.5%, Table 6). Homologous recombination events that converted $1C^n$:$1C^c$ to $2C^n$:$0C^c$ occurred at the same frequency as events converting $1C^n$:$1C^c$ to $0C^n$:$2C^c$ (Table 6).

Fertility and retention of A- and B-genome loci in the unreduced gamete-derived lines

Self-fertile unreduced progeny (those that produced at least one seed in self-pollination bags) had lost significantly fewer B-genome loci (8% less on average) than infertile progeny (Table 7). Self-fertile unreduced progeny had also lost fewer A-genome loci (4% less on average) than infertile progeny, but this difference was not significant (Table 7). In the unreduced gamete-derived population of 55 individuals, 23 were self-fertile, with an average of eight and a maximum of 96 seeds per plant.

Discussion

Overview

The purpose of this study was to evaluate genetic effects on abnormal meiosis in interspecific *B. napus* × *B. carinata* (C^nC^cAB) hybrids. We confirm the previous conclusion that a high proportion of microspore-derived progeny from the C^nC^cAB hybrids were derived from unreduced gametes (Nelson et al. 2009). Presence–absence markers of known genomic locations showed that the frequency of recombination events in the A- and B-genome linkage groups

Table 6 Variation in inheritance of C genome alleles among eight hybrid progeny sets of unreduced microspore-derived individuals from their *B. napus* (AAC^nC^n) × *B. carinata* (BBC^cC^c) hybrid parents (genome complement C^nC^cAB)

Hybrid progeny set[a]	$0C^n$:$1C^c$ or $2C^n$:$1C^c$	$1C^n$:$0C^c$ or $1C^n$:$2C^c$	$2C^n$:$0C^c$	$0C^n$:$2C^c$	$1C^n$:$1C^c$	Total
H1-1	5	4	2	2	19	32
H1-2	13	6	3	10	62	94
H1-4	0	1	2	0	11	14
H2	2	7	4	9	31	53
H3	9	4	6	3	22	44
H4	1	2	3	0	16	22
H5-1	4	9	1	2	45	61
H6	3	3	4	1	8	19
Total (%)	37 (10.9%)	36 (10.6%)	25 (7.4%)	27 (8.0%)	214 (63.1%)	339

Microsatellite marker allele copy number analysis was used on seven markers amplifying C^n, C^c and at least one other allele (from the A and/or B genomes) to identify the ratio of C^n to C^c at seven homologous loci in the progeny. $1C^n$:$1C^c$ is expected if no recombination events have occurred, $0C^n$:$2C^c$ or $2C^n$:$0C^c$ if homologous recombination has occurred, and other ratios if non-homologous recombination or other events resulting in unbalanced segregation of chromosomes have occurred

[a] Hybrid progeny sets varied significantly in distribution of observations across categories ($P = 0.014$, Fisher's exact test for count data with Monte-Carlo simulation)

Table 7 Proportion of A (P_A) and B (P_B) genome loci missing among fertile and sterile individuals in eight *B. napus* (AAC^nC^n) × *B. carinata* (BBC^cC^c) hybrid progeny sets of unreduced microspore-derived individuals

Progeny set	Fertile			Sterile		
	P_A	P_B	*n*	P_A	P_B	*n*
H1-1	0.10	0.13	1	0.21	0.12	4
H1-2	0.05	0.05	12	0.09	0.09	6
H1-4	–	–	0	0.16	0.03	2
H2	0.07	0.00	1	0.14	0.05	7
H3	0.15	0.13	3	0.17	0.24	4
H5-1	0.06	0.05	4	0.15	0.15	5
H6	0.15	0.13	1	0.22	0.16	2
H4	0.27	0.13	2	0.13	0.45	1
Average	0.12[a]	0.09***	Total: 24	0.16[a]	0.16***	Total: 31

Fertile is defined as the ability to produce at least one seed under self-pollination conditions

*** Significant differences in loss of B-genome loci between non-fertile and fertile progeny across progeny sets ($P < 0.001$, REML variance components analysis)

[a] No significant difference in loss of A-genome loci between non-fertile and fertile progeny across progeny sets ($P = 0.18$, REML variance components analysis)

varied between hybrid progeny sets (Table 4). Using dosage-sensitive markers on unreduced microspore-derived progeny, we found that hybrid progeny sets also varied in the pattern of allele loss and duplication between the A, B and C genomes (Table 5) as well for homologous recombination in the C genome (Table 6). Only a proportion of the homoeologous recombination events occurring during meiosis in the interspecific C^nC^cAB hybrids could be detected using our methods, as homoeologous reciprocal translocations could not be detected, and the molecular markers were relatively far apart on the chromosomes. Nevertheless, we have demonstrated genetic effects on the frequency of abnormal meiosis between the *Brassica* A, B and C genomes in *B. napus* × *B. carinata* interspecific hybrids, most likely as a result of allelic variation for control of homologous and non-homologous chromosome pairing.

Genetic control of homologous, homoeologous and paralogous recombination events

Our molecular marker observations of genotypic differences between progeny sets are most likely due to genetic variation present in our population for control of recombination events between regions of homology, primary homoeology (orthologous chromosomes or chromosomal segments resulting from relatively recent genome divergence between the A, B and C genomes (Lagercrantz and Lydiate 1996)) and secondary homoeology (paralogous chromosomes or chromosomal segments resulting from more ancient polyploidisation events). Paralogy in the *Brassica* genomes is well established, with current models proposing a genome-wide triplication event prior to the divergence of the A, B and C genomes (Lagercrantz and Lydiate 1996; Lysak et al. 2005) supported by reports of autosyndesis within each of the three genomes (Armstrong and Keller 1981, 1982; Prakash 1973). Autosyndesis has also been observed within interspecific *Brassica* hybrids between each of the haploid A, B and C genomes (Mason et al. 2010).

Homoeologous recombination is evaluated in our study through duplication/deletion events for alleles amplified by

the same marker (HNRTs, category iii) in Table 5). Paralogous recombination events may also be inferred through duplication or deletion events without a detectable change in homoeologous allele dosage [categories (i) and (ii) in Table 5]. *Brassica* hybrids and synthetics both demonstrate high frequencies of non-homologous pairing via chiasma formation at metaphase I (Harberd and McArthur 1980), and this effect is even more pronounced in early generations of synthetic *B. napus* (Gaeta et al. 2007; Song et al. 1995). A recent study by Nicolas et al. (2009) demonstrated that chromosome rearrangements for some linkage groups in *B. napus* "Darmor" can approximate the number of chiasma observed during metaphase I chromosome pairing, lending weight to the relationship between chiasma formation during meiosis in the parent and detectable chromosome recombination events in progeny. Hence, our marker analysis of unreduced gamete-derived progeny, which revealed high frequencies of non-homologous recombination, supports the molecular cytogenetic observation of high frequencies of multivalent formation in *B. napus* × *B. carinata* hybrids presented in Mason et al. (2010).

Genetic effects on homologous and non-homologous recombination events in the experimental population

Frequency of non-homologous recombination events in the A and B genomes differed significantly between progeny sets (Table 4), and overall loss of A and B genome loci was also different between progeny sets (Table 3). Hence, genotypic differences in meiotic behaviour between C^nC^cAB hybrids were detected by our analysis of linkage groups and missing loci in the A and B genomes (Tables 3, 4). However, significant differences were found in total A- and B-genome loci lost between two C^nC^cAB hybrid progeny sets from different hybrid plants with the same parent varieties (Table 3). This may be due to the heterozygous and heterogeneous nature of the *B. carinata* paternal parent, which may segregate for homoeologous pairing control alleles in parental gametes.

We observed significant genetic effects on the frequency of homologous recombination between the *B. napus* and *B. carinata* C genomes during meiosis (Table 6). Genetic differences in homologous recombination frequency have been observed in *Brassica* relative *Sinapis alba* (Nelson et al. 2005) as well as *B. napus* (Leflon et al. 2006; Nicolas et al. 2009; Sheidai et al. 2006). Our results suggest that homologous C genome chromosomes in *B. napus* × *B. carinata* hybrids pair relatively normally, despite their origins in different species (i.e. C^n and C^c) and the presence of A and B genome univalents. Surprisingly, putative non-homologous recombination events involving the C genome (represented in Table 5 by conversion to one or three C genome alleles at a single locus) were more commonly observed than homologous recombination events (Table 5). Possibly, micro-inversions and other chromosomal rearrangements between the *B. napus* and *B. carinata* C genome linkage groups are responsible for an over-estimation of non-homologous recombination events in this system.

The observed differences in meiotic behaviour in our study are consistent with the findings of Jenczewski et al. (2003) and Liu et al. (2006), who also identified genotypic differences in homoeologous pairing control in haploid *B. napus*, both as the result of a major gene (*PrBn*) and several minor genes. Homoeologous pairing in *B. juncea* (AABB) is also known to be genetically determined (Prakash 1974). Although homoeologous pairing in *B. carinata* has not been similarly analysed, it is reasonable to assume that it is also under genetic control, and that homoeologous pairing control alleles from both the *B. napus* maternal parent and the *B. carinata* paternal parent were expressed in the interspecific (C^nC^cAB) background. Chromosome-specific effects on non-homologous and homologous recombination frequency have also been identified in *B. napus* (Nicolas et al. 2009), and this effect may be amplified in our study due to the probable presence of numerous minor inversions and chromosome rearrangements between the *B. napus* and *B. carinata* C genomes. Despite the complexity of meiotic regulation in *Brassica*, this allelic variation for meiotic behaviour may be exploited by breeders to produce interspecific hybrids with high levels of homoeologous pairing for transferring alleles between genomes, or conversely to produce higher ploidy progeny with little homoeologous exchange and a more stable polyploid genome.

Observation of identical haplotypes in the experimental unreduced gamete-derived population

Within progeny sets there were some progeny with identical haplotypes. These progeny may have resulted from clonal reproduction of microspore-derived embryos (twins), such as observed in *B. napus* microspore culture (Cousin and Nelson 2009) or secondary embryogenesis (Raemakers et al. 1995). To ensure that only unique haplotypes were included, all but one of each haplotype from the same microspore isolation group were removed from the statistical analyses. This may have introduced a conservative bias in our estimates of genetic variation, leading us to underestimate the effect of genotype on non-homologous recombination. Removal of identical individuals which were not derived from clones would increase the overall variation within progeny sets, hence decreasing differences between them. On two occasions identical haplotypes occurred in different microspore isolations, and these progeny were not excluded from analysis as they could not have been derived from secondary embryogenesis or

twinning. The occurrence of identical haplotypes in different microspore isolations was a surprising result, which may be due to selection in microspore culture for favoured homoeologous chromosome pairing events in the interspecific hybrid plants. Highly genotype-specific recombination between individual chromosomes may also be responsible for this effect. However, recent analyses of the effect of *PrBn*, a major homoeologous pairing control gene in *B. napus*, have shown that this locus affects frequency rather than distribution of crossover events during meiosis (Nicolas et al. 2009). Hence, selection of specific haplotypes through the microspore culture process is probably responsible for the prevalence of identical haplotypes.

The effect of loss of A- and B-genome loci on fertility

The fertility of individuals derived from unreduced microspores was higher in those with greater retention of B-genome loci, although we detected no significant effect of A-genome loci on fertility (Table 7). This suggests that retention of the B genome may be more important for maintaining fertility than retention of the A genome in higher ploidy hybrids. This could be due to lower redundancy between the B genome and the A/C genomes in the amphidiploid species, and the greater differentiation of the B genome from the A and C genomes. Our results conform to the expectation that higher levels of homoeologous recombination during meiosis in the C^nC^cAB interspecific hybrid should be associated with reduced fertility in the progeny. However, all individuals were treated with colchicine to double chromosome number and improve self-fertility, but this putative doubling was not confirmed. Therefore, response to colchicine may be a confounding factor in this analysis—it is possible that greater retention of B-genome loci is actually correlated with chromosome doubling in response to colchicine, which may then be responsible for the increased self-fertility observed.

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