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ORIGINAL PAPER

Microspore culture preferentially selects unreduced (2n) gametes from an interspecific hybrid of *Brassica napus* L. × *Brassica carinata* Braun

Matthew N. Nelson · Annaliese S. Mason · Marie-Claire Castello · Linda Thomson · Guijun Yan · Wallace A. Cowling

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Abstract We analysed the products of male meiosis in microspore-derived progeny from a *Brassica napus* (AAC^nC^n) × *Brassica carinata* (BBC^cC^c) interspecific hybrid (ABC^nC^c). Genotyping at 102 microsatellite marker loci and nuclear DNA contents provided strong evidence that 26 of the 28 progeny (93%) were derived from unreduced (2n) gametes. The high level of C^nC^c marker heterozygosity, and parallel spindles at Anaphase II in the ABC^nC^c hybrid, indicated that unreduced gametes were formed by first division restitution. The frequency of dyads at the tetrad stage of pollen development (2.6%) suggested that unreduced gametes were preferentially selected in microspore culture. Segregation of marker alleles in the microspore-derived progeny was consistent with homologous recombination between C^n and C^c chromosomes and homoeologous recombination involving A-, B- and C-genome chromosomes during meiosis in the ABC^nC^c hybrid. We discuss the potential for using microspore culture of unreduced gametes in interspecific hybrids to map *Brassica* centromeres through half-tetrad analysis.

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M. N. Nelson (✉) · A. S. Mason · M.-C. Castello · L. Thomson · G. Yan · W. A. Cowling
Faculty of Natural and Agricultural Sciences,
School of Plant Biology, The University of Western Australia,
35 Stirling Highway, Crawley, WA 6009, Australia
e-mail: mnelson@plants.uwa.edu.au

Introduction

The unique genomic relationships among the six cultivated *Brassica* species are well established, having first been summarised in the Triangle of U more than 70 years ago (Nagaharu 1935) (Supplementary Fig. 1). Crossing allotetraploid *Brassica* species has been proposed as an efficient means of transferring polygenic traits between oilseed *Brassica* crop species; for example Prakash and Chopra (1990) used this approach to transfer pod shatter resistance from *B. juncea* (AABB) to *B. napus* (AACC) via an AABC interspecific hybrid. In the AABC hybrid example, normal homologous recombination is expected between A-chromosomes while a lower frequency of homoeologous recombination is expected between unpaired B- and C-chromosomes. However, these expectations have not been satisfactorily validated using cytogenetic means due to the lack of distinguishing features among *Brassica* chromosomes (Snowdon 2007).

Genotyping progeny using molecular markers with known chromosome locations is currently the most effective means for inferring detailed meiotic chromosome behaviour in interspecific *Brassica* hybrids. Restriction fragment length polymorphism (RFLP) markers have been widely used to detect changes in allele copy numbers associated with homoeologous non-reciprocal translocations (HNRTs) (e.g. Sharpe et al. 1995; Udall et al. 2005). Recently, PCR-based markers have been used to detect HNRTs in *Brassica* interspecific crosses (Leflon et al. 2006; Nicolas et al. 2007) and copy number changes in *Arabidopsis* polyploids (Henry et al. 2006).

Microspore culture (androgenesis) is routinely used in *Brassica* species to produce homozygous doubled haploid lines for breeding and experimental purposes (reviewed by Friedt and Zarhloul 2005). A significant advantage of using

microspore-derived (MD) progeny to study the meiotic process in hybrids is that only a single gamete contributes genetic material to the MD progeny, rather than the union of a male and a female gamete, thus reducing the complexity of genotyping MD progeny. Microspore culture of interspecific *Brassica* hybrids is less common but has been reported for *B. napus* × *B. rapa* (Kubik 1999; Zhou and Scarth 1995) and hexaploid *Brassica* × *Orychophragmus violaceus* (Ge and Li 2006) hybrids.

In this study, we performed microspore culture on an ABC^nC^c interspecific hybrid produced by crossing *B. napus* (AAC^nC^n) with *B. carinata* (BBC^cC^c). Our initial aim was to develop MD progeny with recombinant chromosomes for breeding purposes. However, molecular marker and flow cytometric analyses revealed an unexpected result: that microspore culture preferentially selected first division restitution (FDR)-like unreduced gametes. We discuss the potential of unreduced MD progeny from *Brassica* interspecific hybrids for mapping the genetic location of centromeres.

Materials and methods

Genetic material and hybridity testing

Controlled reciprocal crosses between the doubled haploid *B. napus* cultivar 'Trilogy' (provided by Canola Breeders Western Australia Pty Ltd) and *B. carinata* breeding line '94024.2' (a selection made by Margaret Campbell, The University of Western Australia from accession ATFCC 94024 from the Australian Temperate Field Crops Centre, Horsham, Australia) were performed in controlled environment growth rooms with 16 h photoperiod, a light intensity of approximately 200 μmol/m^2/s and temperature of 18/13°C (day/night). The same conditions were used for growth of F_1 hybrids and MD progeny. Genomic DNA was extracted from fresh leaf tissue from 'Trilogy' and '94024.2' parents, 20 putative F_1 individuals and subsequent MD progeny using a standard CTAB method (Murray and Thompson 1980). Hybridity was tested using the nuclear genome-located microsatellite marker Na10-D09 (Lowe et al. 2004) using the methods detailed below. PCR products were resolved by TBE agarose gel electrophoresis using 4% Agarose 1000 (Invitrogen, Carlsbad, California). Cross progeny were considered true interspecific hybrids when they possessed alleles from both parents.

Microspore culture

Sixty to eighty floral buds of 2–4 mm in length were selected from a confirmed interspecific F_1 hybrid individual ('MC143') and from each parent ('Trilogy' and '94024.2') for separate microspore isolations using standard sterile techniques. Microspore isolation of the F_1 hybrid was replicated three times. Buds were surface-sterilised using 1% sodium hypochlorite (w/v) for 15 min, followed by rinsing with sterile, distilled water. The buds were then gently squeezed to release microspores using a glass rod in 5 mL of half concentration B5 Gamborg medium (Austratec, Kilsyth, Victoria) plus 13% sucrose (pH 5.8). The microspores were then filtered through a 44 μm filter and the volume adjusted to 30 mL with additional media. The microspores were pelleted by centrifugation for 5 min at 215*g*, resuspended in 30 mL of fresh medium then centrifuged again. The microspores were resuspended in 30 mL NLN-13 solution [NLN medium powder (Austratec) plus 13% sucrose, pH 5.8] and the microspore density was determined using a haemocytometer. After 5 min centrifugation at 215*g* the microspores were resuspended in fresh NLN-13 medium to give a density of 2×10^4/mL. 100 μL of 1% activated charcoal was added to 15 mL of the microspore culture. The cultures were kept in the dark and subjected to heat-treatment at 32.5°C for 24 h, and then transferred to a 25°C growth room for 2 weeks. After that, the cultures were shaken for a further week at the same temperature and then finally exposed to light for 1 week.

Plant regeneration

Cotyledonary embryos were transferred to regeneration media at a maximum of 9 embryos per 90 mm plate on B5 medium (Gamborg et al. 1968) plus 2% sucrose, 1.5 mg/L BAP (6-benzylaminopurine), with 0.5 mg/L *trans*-zeatin, 0.4% Phytagel (Sigma–Aldrich, Sydney) and 0.3% agar, and grown at 25°C at 16 h photoperiod in the culture room for 4 weeks to promote regeneration.

Regenerated embryos showing evidence of true leaf growth were transferred to new B5 media containing no hormones, 0.4% Phytagel and 0.3% agar. After 4 weeks the plantlets that had roots were cleaned and directly transferred to soil. The plantlets were grown for 2 weeks in a well-lit culture room at 15°C and then finally transferred to a controlled environment room as described above.

Cytological analyses of pollen and pollen mother cells

Pollen viability of the parents, 3 F_1 hybrids ('MC111', 'MC126' and 'MC148', which were full sibs of 'MC143'), and 28 MD progeny was counted using the aceto-carmine stain method (Yan et al. 1997) on 500 pollen grains from each of four newly opened flowers per plant. Pollen was collected onto a glass slide and a drop of 2% aceto-carmine added. Swollen grains that stained red were counted as viable; shrivelled or malformed, unstained pollen grains were counted as unviable.

Flower buds were collected in Carnoy's II solution (ethanol:chloroform:glacial acetic acid = 6:3:1) and incubated for 24 h at room temperature before being transferred to 70% ethanol for storage at 4°C. Fixed buds of the appropriate size were dissected to remove anthers. A single anther per slide was squashed in FLP orcein stain for meiotic observations of pollen mother cells (PMC's).

Flow cytometry

Flow cytometry methods were based on Dolezel and Bartos (2005). At least three samples per plant, each on different days, were analysed using *Lactuca sativa* 'Grand Rapids' as an internal standard. DNA content (2C) of normal diploid nuclei was determined for *B. napus* and *B. carinata* parents, interspecific F_1 and 28 MD progeny.

Sample preparation was based on the protocol optimised for *Chamerion angustifolium* (Paul Kron and Brian Husband, pers. comm. 2005). New leaves were harvested from all plants to minimise development and metabolic effects of chromatin condensation on DNA staining (Dolezel and Bartos 2005). A sample:standard ratio of 2:1 was used for isolation of nuclei (Arumuganathan and Earle 1991). Sample (100 mg) and standard (50 mg) were finely chopped with 2 mL of ice-cold nuclei isolation buffer (Bino et al. 1992; adjusted to pH 6.8 instead of 8.0). Leaf tissue was suspended in the nuclei isolation buffer using a 1 mL pipette with cut tip to take up and release the homogenate 25 times; with the final release strained through 30 µm nylon mesh (Sefar Filter Specialists, Malaga, Western Australia) into a chilled tube. The homogenate was then centrifuged for 15 min at 100*g*. The pellets were resuspended in 400 µL freshly-made staining solution (Arumuganathan and Earle 1991) and stored in the dark for up to 2 h.

Propidium iodide (PI)-stained nuclei were analysed on a Becton-Dickinson FACSCalibur flow cytometer (BD Biosciences, Australia), using a 488 nm Argon laser to excite the PI fluorochrome, and FL-2 detector with a 585/42 band pass filter. The primary threshold was forward scatter, the secondary threshold was PI fluorescence, and all parameters were linear measures. A doublet gate was set on FL-2 to discriminate single nuclei. Samples were run on low pressure and 10,000 nuclei were counted within the doublet gate. The *L. sativa* 'Grand Rapids' histogram peak was adjusted to approximately 200 on the FL-2 area axis at the beginning of each acquisition session. Histogram peak positions were analysed using CellQuest Pro software (Becton, Dickinson and Company 2004). Only samples with coefficients of variation less than six were included for analysis. Sample 2C DNA was calculated and mean DNA amount was used to calculate ploidy (*x*) based on the mean DNA content of the interspecific hybrid (ABC^nC^c), which was assumed to be 4*x*.

Molecular marker characterisation of MD progeny

The primer pair sequences of 79 A/C-genome and 48 B-genome microsatellite markers with known genomic locations in *B. napus* and *B. juncea* (respectively) were provided by A. Sharpe and D. Lydiate (Agriculture and AgriFood Canada Saskatoon Research Centre, Saskatoon; pers. comm.; for more information, see http://brassica.agr.gc.ca). The map location of six previously unmapped loci detected by primers pairs sN2552 and sORH13 (A-genome loci) and sR9555, sN1988, sN11722 and sN11707 (B-genome loci) were inferred based on conserved syntenic relationships reported by Lagercrantz and Lydiate (1996) and Parkin et al. (2003). Two further primer pairs (Na12-G05 and Ra2-E04) were developed by Lowe et al. (2004); their map locations were reported by Chen et al. (2007). All markers were screened on the six species of U's Triangle (Supplementary Fig. 1) including the *B. napus* and *B. carinata* parents used in this experiment. The most informative markers were selected for characterisation of MD progeny of the interspecific hybrid (ABC^nC^c).

Microsatellite markers were amplified in 25 µL reactions with the final concentrations: 2 ng/µL DNA template, 1 × PCR buffer (Promega), 0.04 U/µL Taq Polymerase, 2 mM $MgCl_2$, 200 µM of each dNTP and 0.2 µM of each primer. PCR was performed using an Eppendorf Mastercycler with the following thermal cycling: initial denaturing of 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 60 s at 72°C; with final extension at 72°C for 7 min (B-genome markers) or 15 min (A/C-genome markers). The 5′ nucleotide of the forward primers of the A/C-genome markers were fluorescently labelled using 6FAM (Illumina, San Diego), PET, NED or VIC (Applied Biosystems, Scoresby, Victoria) and amplification products assayed on an AB3730*xl* capillary DNA sequencer with fragment analysis conducted using Genemapper 3.7 software (Applied Biosystems). This assay method allowed resolution of small sizes differences between C-genome alleles from *B. napus* (C^n) and *B. carinata* (C^c) and the scoring of the presence or absence of A- and/or B-genome alleles. Most A/C-genome markers used to screen MD progeny (indicated in Supplementary Table 1) showed consistent relative peak heights at homoeologous loci and could be used to detect changes in relative allele copy numbers in the MD progeny. Markers that detected single loci on the A- or B-genome were scored for presence or absence of bands detected by standard TBE agarose gel electrophoresis using 2% agarose gels stained with ethidium bromide.

Results

Interspecific crossing and microspore culture

Twenty seeds produced from reciprocal crosses between *B. napus* 'Trilogy' and *B. carinata* '94024.2' were germinated and plants screened for hybridity using microsatellite marker Na10-D09. When 'Trilogy' was the female parent, all 12 progeny tested were confirmed as true interspecific hybrids. When '94024.2' was the female parent, all eight progeny tested appeared to be self-progeny or matromorphs. One confirmed F_1 hybrid ('MC143') was selected for subsequent microspore culture and other confirmed hybrids were used as controls. Three microspore isolation experiments from hybrid 'MC143' yielded 57 embryos, of which 45 plantlets were deflasked and 28 plants survived beyond flowering; these latter MD progeny were used in subsequent analyses. Single microspore isolations of 'Trilogy' and '94024.2' parental controls yielded 100 and 48 embryos, respectively.

Transmission of A-, B- and C-genome chromosomes inferred by microsatellite marker genotyping

Of the 129 microsatellite primer pairs screened for suitability in the six species of U's Triangle (Supplementary Fig. 1), 84 primer pairs detected 40, 36 and 26 loci on A-, B- and C-genome chromosomes, respectively, and these primer pairs were used to genotype the 28 MD progeny along with parental and F_1 controls; see Supplementary Table 1 for all genotyping data. Figure 1 shows electropherograms for a typical marker that detected homoeologous loci on the A- and C-genomes, and which could be used to determine relative copy numbers of alleles at homoeologous loci (see Fig. 1 legend for further explanation). Of 26 markers that detected C-genome loci, 20 were used to estimate relative allele copy number (indicated in Supplementary Table 1).

Transmission frequencies of loci on two chromosomes (A6 and B8) in the MD progeny did not deviate significantly from 50% as expected for normal reduced (n) gametes from the ABC^nC^c hybrid (Chi-square test $P > 0.05$). However, loci on all other A- and B-genome chromosomes showed allele transmission frequencies above 80%, significantly greater than the expected 50% (Chi-square test $P < 0.01$). Alleles at loci on the same chromosome in the A or B genome had similar transmission frequencies (Supplementary Fig. 2a and b). Of the 52 C-genome marker alleles, 40 were transmitted at frequencies significantly greater than 50% (Chi-square test $P < 0.01$) and there was considerable variation in allele transmission frequency among loci both within and between C-chromosomes (Supplementary Fig. 2c).

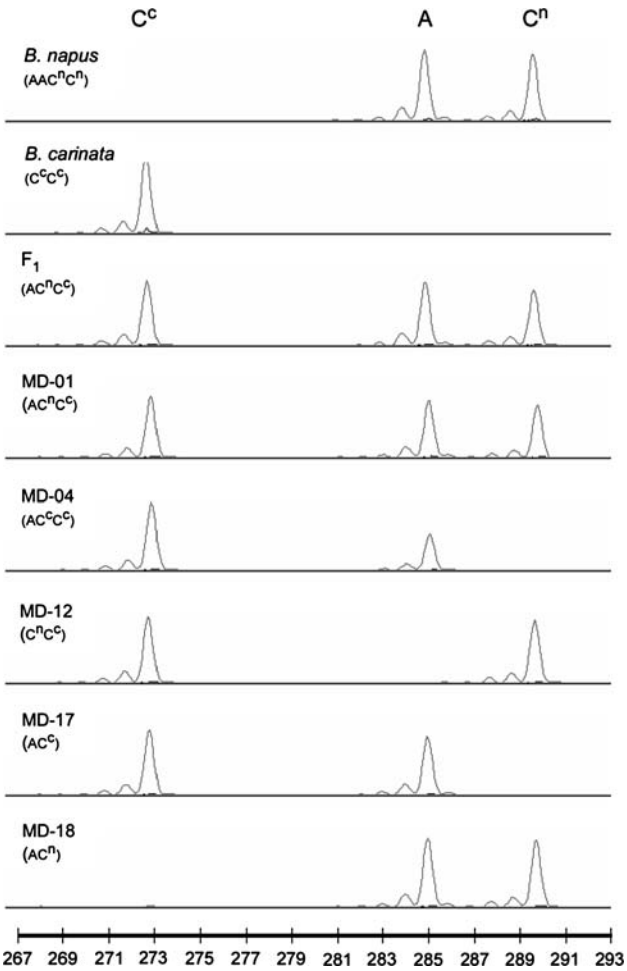

Fig. 1 Electropherogram of microsatellite marker sR94102 in *B. napus* 'Trilogy' and *B. carinata* '94024.2' parents, interspecific F_1 hybrid and five microspore-derived (MD) progeny. The horizontal scale indicates estimated amplification product sizes in base pairs (bp). The peaks at 273 bp and 290 bp correspond to the C-genome locus on linkage group C2 of *B. carinata* (C^c) and *B. napus* (C^n), respectively; the peak at 285 bp corresponds to the homoeologous A-genome locus on linkage group A2 of *B. napus*. The relative peak heights of homoeologous loci were used to determine the allelic composition of MD progeny (shown in *parentheses*). For example, MD-04 (derived from an unreduced gamete) and MD-17 (derived from a reduced gamete) possessed the same alleles but in different proportions: MD-04 had a ratio of $2C^c$:1A alleles while MD-17 had a ratio of $1C^c$:1A alleles

Normal Mendelian segregation of C-genome marker alleles—where the MD progeny possesses one parental allele type at each locus—was observed in only two MD progeny ('MD-17' and 'MD-18'; Supplementary Table 1), consistent with their derivation from reduced (n) gametes. The remaining 26 lines were frequently heterozygous at most C-genome loci (70.2% overall), consistent with their derivation from unreduced (2n) gametes produced by an FDR-like mechanism.

Ploidy status of MD progeny

Flow cytometry

The nuclear DNA content from flow cytometry (2C ± SE) of *B. napus* and *B. carinata* parents was estimated to be 2.52 ± 0.01 pg and 2.60 ± 0.01 pg, respectively, with the interspecific hybrid (ABC^nC^c) intermediate at 2.58 ± 0.01 pg. The nuclear DNA content of MD progeny was expressed as DNA ploidy level (*x*) compared with the ABC^nC^c hybrid (set at $4x = 2.58$ pg). On this basis, the DNA ploidy level of the MD progeny ranged from 2.4*x* to 8.3*x*. MD progeny were classified into three major groups, based on a comparison of ploidy level (estimated as above) and marker-based chromosomes counts: (i) reduced gametes (close to 2*x*); (ii) unreduced gametes (close to 4*x*); and (iii) unreduced gametes that had apparently undergone post-meiotic chromosome doubling (close to 8*x*) (Fig. 2). One unreduced progeny (MD-20) was possibly a chimaera with an average of 5.5*x* (Fig. 2).

Cytological confirmation of unreduced gamete formation in the ABC^nC^c hybrid

The frequency of dyads at the tetrad stage of pollen development in the ABC^nC^c hybrid was 2.6% (17 dyads out of 643 cells counted) compared to 0/1119 for parent *B. carinata* '94024.2' and 0/630 for parent *B. napus* 'Trilogy'. We also observed what appeared to be parallel spindles at Anaphase II in anthers of the ABC^nC^c hybrid (Fig. 3).

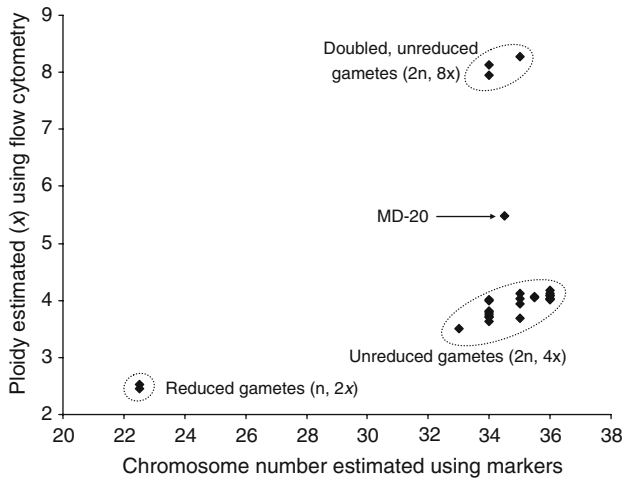

Fig. 2 Chromosome complements estimated using microsatellite markers versus ploidy level (*x*) estimated by flow cytometry in *Brassica napus* 'Trilogy' (*black square*), *B. carinata* '94024.2' (*black circle*), interspecific F_1 hybrid (*black triangle*) and 28 F_1 MD progeny (MD-numbers) (*black diamond*). Three classes of MD progeny are putatively defined: reduced, unreduced and spontaneously doubled, unreduced gametes. MD-20, a putatively chimaeric line, was intermediate between the unreduced and doubled unreduced classes of lines

Plant morphology, pollen viability and seed set

The morphology of the MD progeny varied widely, with some resembling either the *B. napus* or *B. carinata* parents, and others intermediate between the parents. Purple pods, which were characteristic of *B. carinata* '94024.2', were present in approximately half of the MD progeny.

Pollen viability varied widely between the parents, interspecific hybrid and MD progeny. The two parent lines showed close to 100% pollen viability while the interspecific hybrid had 16% pollen viability. MD progeny assigned to the 2*x* group (Fig. 2) (MD-17 and MD-18) had no visible anthers and therefore no viable pollen. MD progeny assigned to 4*x* group (Fig. 2) had pollen viabilities ranging from 0 to 75%. MD progeny in the 8*x* category (Fig. 2) had 83–92% viable pollen. Eight of the MD progeny produced viable seed upon selfing, ranging from 0 to 5 seed per progeny (average = 0.71 seeds per MD progeny). There was a moderate correlation between pollen viability and DNA content ($r = 0.66$, $P < 0.001$). There were also moderate correlations between pollen viability and number of self-seed ($r = 0.53$, $P < 0.005$) and between DNA content and number of self-seed ($r = 0.57$, $P < 0.005$).

Marker segregation in reduced and unreduced MD progeny

Evidence for homologous recombination

Homologous recombination between the C^n and C^c chromosomes (derived from *B. napus* and *B. carinata*, respectively) manifested itself differently in MD progeny derived from reduced and unreduced gametes. MD progeny derived from reduced gametes lines (MD-17 and MD-18) showed homologous recombination in five and seven C-chromosomes, respectively, based on changes in parental allele types along a chromosome (Supplementary Table 1). In unreduced MD progeny, homologous recombination between a locus and a centromere was inferred by homozygosity (C^n/C^n or C^c/C^c) at that locus. The absence of recombination (or the occurrence of two crossovers) between a locus and a centromere was inferred by heterozygosity (C^n/C^c) at that locus. Among the 26 unreduced MD progeny, there were between 2 and 5 recombinant C-chromosomes per line (average of 3.1) (Supplementary Table 1).

Evidence for homoeologous recombination

Homoeologous recombination at meiosis in the ABC^nC^c hybrid was inferred from marker-defined duplications and deletions in MD progeny. Deletions were easy to detect in the A-, B- and C-genome chromosomes and all 102 marker loci were useful for this purpose. Supplementary Table 2 summarises the number of A-, B- and C-genome chromosomes

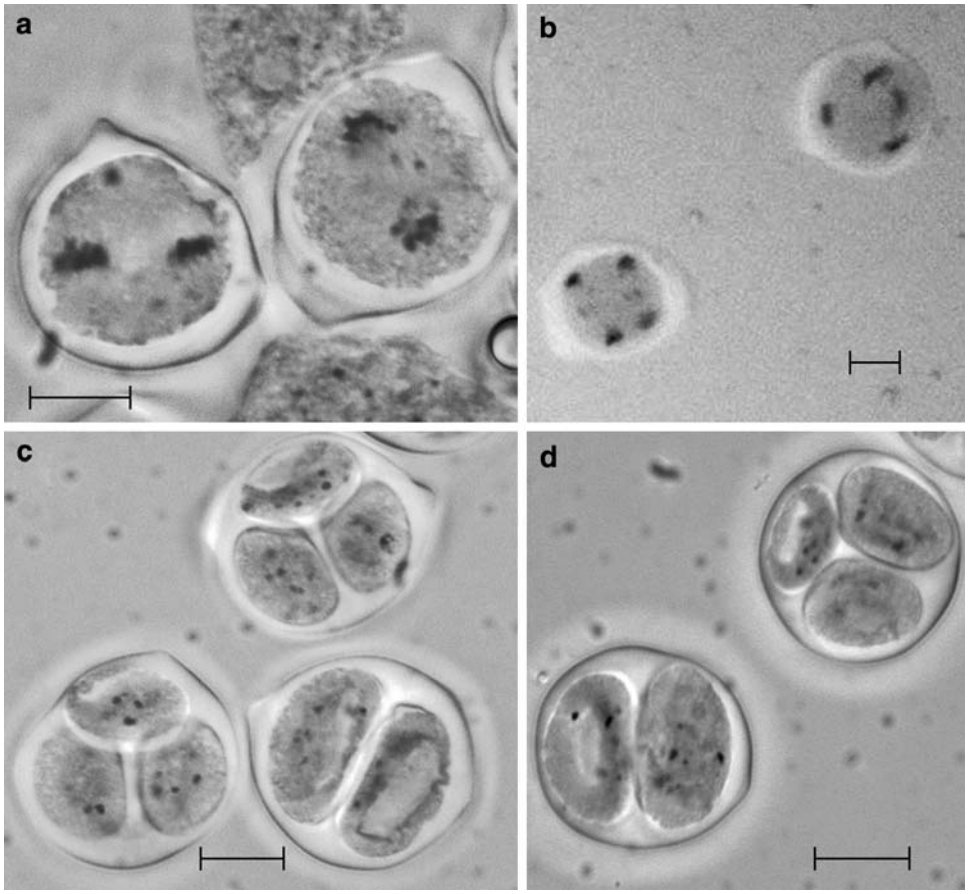

Fig. 3 Micrographs of meiotic stages in pollen mother cells (PMC's) in an interspecific F_1 hybrid produced by crossing *Brassica napus* 'Trilogy' and *B. carinata* '94024.2'. **a** Two PMC's with one at Metaphase II stage showing parallel spindles (*left*) and one at Anaphase I showing laggards (*right*); **b** Two PMC's at Anaphase II with one showing normal spindle configuration (*top-right*) and one showing parallel spindles (*bottom-left*); **c** PMC's at tetrad stage showing one dyad (*bottom-right*) and two normal tetrads; **d** PMC's at tetrad stage showing one dyad (*bottom-left*) and one tetrad (*top-right*). *Scale bars* represent 10 μm

showing evidence of deletions at one or more loci detected in the 26 unreduced MD progeny. Deletions in the unreduced MD progeny may have been caused by homoeologous pairing or by aneuploidy (note the laggards observed in PMCs during Anaphase I in Fig. 3a). Deletions of A- and B-genome loci in progeny MD-17 and MD-18 (derived from reduced gametes) were not necessarily related to homoeologous interactions or aneuploidy, as 50% transmission of these loci was expected.

Twenty markers were suitable for detecting changes in allele copy numbers (indicated in Supplementary Table 1) and were used to identify allele duplications and deletions. Figure 4 shows an example of such a marker: sR12387 detected homoeologous loci on chromosomes A7 and C6. The interspecific F_1 hybrid (ABC^nC^c genome) had an allele ratio of 1A:2C, as did the majority of unreduced MD progeny; however, 5 MD progeny had the allele ratio of 2A:1C indicative of an HNRT resulting from homoeologous pairing between chromosomes A7 and C6. In total, 50 chromosomes in 26 unreduced MD progeny showed evidence of allele duplication (Supplementary Table 2). Some of the duplications coincided with clear deletions at corresponding homoeologous marker loci while others did not. Pairs of homoeologous chromosomes that showed simultaneous duplications/deletions in the A- and C-genome of one or more progeny were: A1/C1, A2/C2, A3/C3, A5/C4, A6/C7, A7/C6, and A10/C9 (Supplementary Table 2).

One marker (sN11707) that detected homoeologous loci on chromosomes A1 and C1 also detected a previously unmapped locus in the B-genome. Assuming that this locus was homoeologous to A1 and C1, then this locus was probably located on chromosome B5 judging by the syntenic relationships among the *Brassica* genomes reported by Lagercrantz and Lydiate (1996). There was a clear duplication/deletion relationship between chromosomes C1 and B5 (data not presented)—the only clear example of an HNRT involving the B-genome.

Discussion

The formation of unreduced (2n) gametes has been observed in a number of plant genera including *Brassica* (Bretagnolle and Thompson 1995; Heyn 1977), and is thought to be an important mechanism in the formation of polyploids (Leitch and Leitch 2008). At the tetrad stage of pollen development in the ABC^nC^c hybrids, we observed relatively high frequencies of dyads (2.6%), a signature of unreduced male gamete formation (Bretagnolle and Thompson 1995). This far exceeded the level of dyad

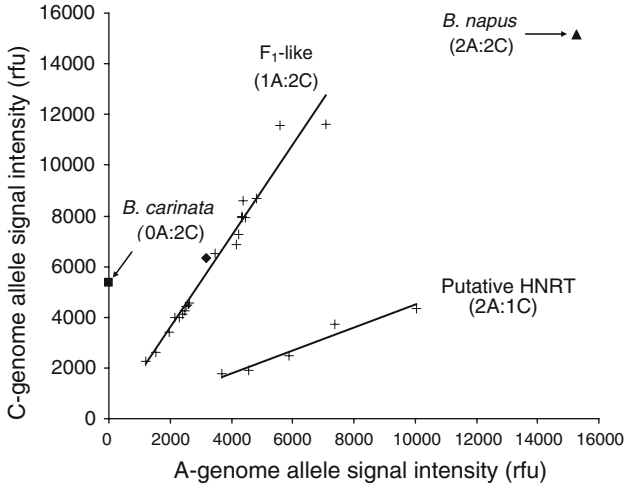

Fig. 4 Detecting putative homoeologous non-reciprocal translocations (HNRT) in unreduced MD progeny from an ABC^{n}C^{c} genome hybrid using relative fluorescent signal intensities at homoeologous loci. Fluorescent signal intensities for VIC-labelled sR12387 marker alleles for homoeologous A7 and C6 loci were plotted for parents (*black triangle*, *B. napus* 2A:2C; *black square*, *B. carinata* 0A:2C), F_1 (*black diamond*, 1A:2C) and 2n MD progeny (*plus* 1A:2C or 2A:1C). Linear trendlines intersecting at the origin are shown. rfu = relative fluorescence units measured using an AB3730*xl* capillary sequencer

formation in the *B. napus* and *B. carinata* parents, where dyads were not detected in more than 1,700 cells counted. More remarkable was the predominance (93%) of F_1 MD progeny that were derived from unreduced gametes, which suggests that microspore culture acts as a selection medium for unreduced male gametes from this hybrid. To our knowledge, this is the first report of this phenomenon and it could provide a novel approach for producing large numbers of progeny from unreduced male gametes. The conventional approach for obtaining progeny from unreduced gametes is to cross plants with differing ploidy levels, as has been done extensively in crosses between diploid and tetraploid *Solanum* species (Douches and Quiros 1988; Park et al. 2007). The advantage of using microspore culture over interploidy crossing is that only a single unreduced gamete contributes genetic material to the progeny, making interpretation of marker genotyping more straightforward.

We did not investigate the genetic or developmental factors that may have given rise to this apparent selection for unreduced gametes through the microspore culture process. However, one may hypothesise that the higher transmission of chromosomes from the A-, B- and C-genomes in unreduced gametes (4*x* nuclear DNA content or 100% of F_1 alleles in the absence of homologous or homoeologous chromosome interactions) compared to reduced gametes (1*x*–3*x* nuclear DNA content, or on average 50% of F_1 alleles in the absence of homoeologous chromosome interactions) may have given a selective advantage to unreduced gametes because they stand a lower chance of losing genes required for successful embryo and plant development in culture.

We found strong molecular marker (high frequency of heterozygosity) and cytological (observation of dyads and parallel spindles) evidence for an FDR-like mechanism in the F_1 interspecific hybrid giving rise to unreduced gametes. These FDR MD progeny could be used for genetic mapping of *Brassica* C-genome centromeres using the half-tetrad analysis approach (Douches and Quiros 1988; Park et al. 2007). The precise genetic location of functional centromeres is unknown for *Brassica* chromosomes, although the approximate locations of some centromeres have been estimated by cytogenetic analyses (Snowdon et al. 2002; Ziolkowski et al. 2006), synteny-based comparisons with *Arabidopsis thaliana* and the deduced ancestral karyotype of *Brassica* species (Parkin et al. 2005; Schranz et al. 2006), and clustering of centromeric motif-based markers (Pouilly et al. 2008). Firm knowledge of centromere locations would shed light on chromosome evolution in *Brassica* species, assist in linking genetic and physical maps and help sequence pericentromeric regions of *Brassica* chromosomes.

We found strong evidence of homologous recombination between all nine pairs of C-genome chromosomes in the ABC^{n}C^{c} hybrid, as inferred from segregation of *B. napus* and *B. carinata* derived alleles in progeny derived from both reduced and unreduced gametes (Supplementary Table 1). This is useful information for both theoretical and applied purposes. The theoretical contribution of this finding is that this is the first molecular marker segregation data to support the long-held view that the C-genomes of *B. napus* and *B. carinata* are well conserved at the gross structural level (Bohuon et al. 1996; Prakash and Chopra 1992; UN 1935). Denser marker genotyping will be required to confirm fine-scale structural conservation of the C-genome of *B. carinata* and *B. napus*. The applied contribution of this finding is that the assumption of Prakash and Chopra (1992) of free exchange of alleles between the C-genome chromosomes of *B. napus* and *B. carinata* in ABC^{n}C^{c} hybrids has been verified and this holds promise for transferring polygenic traits between the C-genomes of these species.

We observed duplications and deletions of marker loci on most A-, B- and C-genome chromosomes (Supplementary Table 2). Some markers were sufficiently informative to detect simultaneous duplication at one locus and deletion at another homoeologous locus (Fig. 4), a signature of HNRT. Such markers provided unambiguous evidence of HNRTs involving seven A-genome and seven C-genome chromosomes, and between C1 and B6. Notably, we found no evidence of HNRTs involving A- and B-genome

chromosomes, which was unexpected given the A- and B-chromosomes were unpaired in the ABC^nC^c hybrid.

Many A/C-genome markers detected allele duplications without showing deletions at the corresponding homoeologous loci (and vice versa) (Supplementary Table 2). It is possible that these were also the result of HNRT involving B-genome chromosomes since few of the markers that detected B-genome loci also detected A- or C-genome loci. It is also possible that some of the observed allele deletions were due to loss of chromosomes resulting from other meiotic abnormalities such as irregular pairing and lagging chromosomes, which we regularly observed during the meioses of the ABC^nC^c hybrid (e.g. Fig. 3a). Our experimental approach was unable to detect homoeologous reciprocal translocations (HRTs) because such events do not result in changes of allele copy number and so homoeologous interactions likely occurred at a greater frequency than our data suggest.

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