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ORIGINAL PAPER

Doubled haploids of novel trigenomic *Brassica* derived from various interspecific crosses

X. X. Geng · S. Chen · I. A. Astarini · G. J. Yan · E. Tian · J. Meng · Z. Y. Li · X. H. Ge · M. N. Nelson · A. S. Mason · A. Pradhan · W. J. Zhou · W. A. Cowling

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Abstract To develop doubled haploid (DH) mapping populations of hexaploid *Brassica*, 10 F_1 hybrids derived from crosses between allohexaploid *Brassica* parents were evaluated in this study. The allohexaploid *Brassica* parents were selfed progenies of unique interspecific crosses between *Brassica rapa* (genome AA) × *B. carinata* (BBCC), *B. nigra* (BB) × *B. napus* (AACC), and a complex cross between *B. juncea* (AABB), *B. napus* and *B. carinata*, with relatively stable chromosome number (2n = 54). Hexaploid status and chromosome behavior during meiosis I in four promising F_1 hybrids were assessed using microscopy and flow cytometry, and progeny were obtained following microspore culture. Hybrids H11-2 and H16-1 demonstrated higher amenability for embryo generation, plantlet regeneration, and frequency of production of DH microspore-derived progeny of hexaploid DNA content (6*x*) compared to hybrids H08-1 and H24-1. A total of 370 6*x* DH progeny were selected out of 693 plantlets from H11-2, 241/436 from H16-1, 23/54 from H08-1, and 21/56 from H24-1. DH progenies of hybrids H11-2 and H16-1 were then designated as promising mapping populations of a new hexaploid *Brassica* species.

Keywords Hexaploid *Brassica* · Microspore culture · Flow cytometry · Interspecific hybridization · Doubled haploid

Abbreviations

ANOVA	Analysis of variance
DH	Doubled haploid
MD	Microspore-derived
PMC	Pollen mother cells
PI	Propidium iodide
RFLP	Restriction fragment length polymorphism
SSR	Simple sequence repeats

Introduction

Brassica crops play important roles in agriculture, from highly diverse vegetables to oilseed crops. Natural polyploidisation among the three diploid species (*Brassica oleracea*, *B. nigra* and *B. rapa*) produced three allotetraploid agricultural species (*B. carinata*, *B. juncea*, and

X. X. Geng and S. Chen: equal first authors.

X. X. Geng · W. J. Zhou (✉)
Institute of Crop Science and Zhejiang Key Laboratory of Crop Germplasm, Zhejiang University, Hangzhou 310058, China
e-mail: wjzhou@zju.edu.cn

X. X. Geng · S. Chen · I. A. Astarini · G. J. Yan · M. N. Nelson · A. S. Mason · A. Pradhan
School of Plant Biology, The University of Western Australia, Crawley, WA 6009, Australia

S. Chen · G. J. Yan · M. N. Nelson · W. A. Cowling
The UWA Institute of Agriculture, The University of Western Australia, Crawley, WA 6009, Australia

I. A. Astarini
Universitas Udayana, Kampus Bukit Jimbaran, Bali, Indonesia

E. Tian · J. Meng · Z. Y. Li · X. H. Ge
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China

Present Address:
A. S. Mason
Centre for Integrative Legume Research and School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

B. napus), as described in the Triangle of U (U 1935). The diploid *Brassica* species contain three distinct genomes: *Brassica rapa* (AA genome), *B. nigra* (BB genome) and *B. oleracea* (CC genome) (U 1935). Each species is genetically diverse and has its own specific adaptations. For example, *B. rapa* is very genetically diverse and has many useful traits, such as its strong resistance to humidity and cold tolerance and its short growth period in subtropical climates, but relatively low grain yield and disease susceptibility limits its application (Kimber and McGregor 1995).

Polyploidisation is an important feature of plant evolution and speciation (Levin 2002; Bennett 2004; Soltis and Soltis 1995). More than 70 % of angiosperms have gone through one or more genome duplication events during the process of evolution (Masterson 1994). The three *Brassica* diploid species naturally combined in pairwise fashion to form three allotetraploid species, namely *B. juncea* (AABB), *B. carinata* (BBCC) and *B. napus* (AACC). However, no allohexaploid *Brassica* species are known in nature. Recently, it was proposed that an allohexaploid *Brassica* species could be formed and tested for tolerance to a range of environmental conditions (Chen et al. 2011), based on the observation that many allopolyploid species are more widely adaptable than their lower ploidy parent species (Murphy et al. 1995). For example, bread wheat (*Triticum aestivum*), a hexaploid crop with A, B and D genomes, is more widely adapted than its lower ploidy relatives, and is now grown from the tropics to the extreme latitudes of agricultural production (Dubcovsky and Dvorak 2007; Leitch and Leitch 2008). Oats are also a hexaploid crop species (*Avena sativa* L., genome AACCDD) (Feldman 2001). Chen et al. (2011) hypothesized that an allohexaploid *Brassica* would also have greater buffering capacity against the shock of climate change and adaptation to a wider range of heat-stressed, drought-stressed, toxic or saline land than their diploid or tetraploid relatives.

Recently, unique allohexaploid *Brassica* lines have been synthesized independently in Australia and China through different interspecific hybridization approaches. Two allohexaploid *Brassica* populations were developed in China from interspecific crosses between *B. rapa* and *B. carinata*, and these populations were advanced 4–5 generations with selection for increasing stability (Li et al. 2005a, b; Tian et al. 2010; Ge et al. 2009). In Australia, allohexaploid *Brassica* was produced through the cross between *B. nigra* and *B. napus* (Pradhan et al. 2010) and also through crosses among the allotetraploid species *B. juncea*, *B. napus* and *B. carinata* (Mason et al. 2012). These allohexaploid *Brassica* from different sources provide invaluable germplasm to breed a diverse but stable hexaploid species. Hexaploid *Brassica* showed instability in chromosome number during selfing, and progenies were selected for "normal" chromosome pairing and segregation over several selfing generations (Tian et al. 2010). This is a similar issue to that experienced in the synthetic allohexaploid crop triticale (AABBRR), the stability of which was improved greatly in later generations following artificial allopolyploidisation from different genetic backgrounds (Larter and Gustafson 1980).

We chose diverse parental *Brassica* material with hexaploid chromosome status from various sources in China and Australia (Li et al. 2005a, b; Tian et al. 2010; Ge et al. 2009; Pradhan et al. 2010; Mason et al. 2012) for intercrossing and selection of potentially stable hexaploid *Brassica* populations. The current research was undertaken to establish DH populations for future genetic mapping of *Brassica* hexaploids.

Microspore culture is important for genetic and genomic research on *Brassica* species (Xu et al. 2007; Ferrie and Mollers 2011). While genetic variation exists within the allotetraploid *B. napus* for production of DH progeny through microspore culture, various techniques have improved embryo recovery and diploidisation from microspore culture (Ahmadi et al. 2012; Takahira et al. 2011). In some diploid *Brassica* species such as *B. oleracea*, it is more difficult to obtain embryos (Winarto and da Silva 2011). Colchicine is most widely used for chromosome doubling (Zhou et al. 2002a, b; Mohammadi et al. 2012). DH populations derived from microspore culture have been widely used to establish genetic linkage maps of allotetraploid *B. carinata* and *B. napus* (Guo et al. 2012; Raman et al. 2012). Ferreira et al. (1994) used a DH population from microspore culture to establish a RFLP linkage map of *B. napus*. Yu et al. (2009) constructed an SSR-based genetic linkage map of diploid *B. rapa* using a DH population. This is the first report of microspore culture on hexaploid *Brassica* to establish DH populations for genetic mapping.

Materials and methods

Plant materials

Allohexaploid *Brassica* parents were derived from several interspecific crosses between *B. rapa* (AA) and *B. carinata* (BBCC) (Li et al. 2005a, b; Ge et al. 2009), *B. nigra* (BB) and *B. napus* (AACC) (Pradhan et al. 2010) and complex crosses among the allotetraploid species *B. juncea* (AABB), *B. napus* and *B. carinata* (Mason et al. 2012) (Table 1).

A total of 18 plants were selected for crossing from 4 different sources of allohexaploid plants with 30 % or higher pollen viability and the expected number of 54 chromosomes in somatic tissue as determined by microscopic examination. Each selected plant was numbered and crossed in various

Table 1 The origins and chromosome numbers of allohexaploid parents used in development of 10 F_1 hybrids, and the chromosome numbers, pollen viability and number of embryos per F_1 bud formed in microspore culture in preliminary experiments

Hybrid code	Parent code [a]		Interspecific crosses used to develop parents[b]		Chromosome number of cross parent[c]		Chromosome number of F_1 hybrid[c]	Pollen viability of F_1 hybrid[d] (%)	No. embryos per floral bud formed in microspore culture (preliminary experiments)	Selected for mapping population development by microspore culture
	Maternal parent	Paternal parent	Maternal parent	Paternal parent	Maternal parent	Paternal parent				
H08-1	C21-4	M07	B.r. × B.c.	(B.j. × B.nap.) × B.c.	54	na	54	23.8 gf	na	Yes
H11-2	C23-3	SP28	B.r. × B.c.	(B.j. × B.nap.) × B.c.	54	na	54	61.2 cd	12.8	Yes
H16-1	7H170-1	Y54-2	B.r. × B.c.	B.nap. × B.nig.	54	54	54	47.3 de	6.5	Yes
H24-1	Y54-2	C26-3	B.nap. × B.nig.	B.r. × B.c.	54	54	54	27.0 fg	15.2	Yes
H01-6	C23-4	C26-1	B.r. × B.c.	B.r. × B.c.	54	54	53	86.0 ab	16.4	
H02-4	C23-1	7H183-3-2	B.r. × B.c.	B.r. × B.c.	54	54	54	20.8 g	na	
H04-6	7H183-3-3	C23-1	B.r. × B.c.	B.r. × B.c.	54	54	54	95.7 a	1.6	
H08-4	C21-4	M07	B.r. × B.c.	(B.j. × B.nap.) × B.c.	54	na	50	54.5 cde	na	
H13-1	SP28	7H067-3-4	B.j. × B.nap. × B.c.	B.r. × B.c.	na	54	45	39.5 ef	na	
H18-1	7H183-3-4	Y54-2	B.r. × B.c.	B.nap. × B.nig.	54	54	53	71.7 bc	1.0	

[a] Parent codes starting with "7", "C" and "Y" are from references (Tian et al. 2010; Ge et al. 2009; Pradhan et al. 2010), respectively. M07 and SP28 are from reference (Mason et al. 2012)

[b] *Brassica* species codes in interspecific crosses: B.r. = *B. rapa*; B.c. = *B. carinata*; B. nap. = *B. napus*; B. nig. = *B. nigra*; and B. j. = *B. juncea*

[c] Chromosomes were counted in 3–5 ovary somatic cells per parent plant growing in the glasshouse and F_1 hybrid plant cuttings growing in a phytotron

[d] Pollen viability was based on 3 young emerged flowers from F_1 hybrid plant cuttings growing in a phytotron, with 600 pollen grains counted from each flower. Means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by Tukey's Honest Significant Differences test

na not available

combinations with other selected plants. Several flowers on each plant were emasculated and pollinated in each cross. Crosses yielded a total of 0–66 hybrid seeds. From 22 successful crosses, 100 F_1 hybrid plants were grown together with 18 parental lines in the glasshouse at The University of Western Australia in 2010 to evaluate hybrid vigour and agronomic suitability. One F_1 seed per cross was used to grow as a mother plant for microspore culture.

The parent allohexaploids and their F_1 hybrid plants were grown in the glasshouse for chromosome counting and pollen viability assessment (Table 1). From 100 F_1 hybrids formed among these sources of *Brassica* allohexaploids, 10 hybrids with diverse pedigree, high parental chromosome number, relatively high pollen viability and improved seed-setting ability were chosen for preliminary observation in microspore culture. Cuttings were made from these 10 F_1 hybrids and were grown in a phytotron at 18 °C/13 °C (day/night) with a 16 h photoperiod at a light intensity of approximately 500 μmol m^{-2} s^{-1} for a second round of microspore culture, chromosome counting and pollen viability (Table 1).

Cytological observations

Chromosome number at mitotic metaphase

The chromosome numbers of 10 F_1 *Brassica* hybrid plants and microspore-derived (MD) progenies were determined in somatic tissue on the young ovaries of new buds, at mitotic metaphase. At least 3 young ovaries per F_1 were collected, treated with 2 mM 8-hydroxyquinoline for 4 h and then fixed in a mixture of ethanol: acetic acid (3:1) overnight, transferred to fresh fixative mixture and stored in freezer until use. Cells were checked for chromosome number following the method of Li et al. (Larter and Gustafson 1980; Li et al. 1995). Tissue was hydrolyzed in 1 N HCl at 60 °C for about 8 min, squashed in a drop of 10 % modified carbol fuchsin and the chromosome number was recorded at mitotic metaphase in 3–5 somatic cells. The highest number was chosen as the final chromosome number.

Chromosome pairing during meiosis

Meiosis was observed in pollen mother cells (PMC) in young anthers from the terminal inflorescence buds, following a modification of Li et al. (1995). More than 3 young buds (approximately 1.5 mm in length) were collected on ice between 9 a.m. and 10 a.m. in the morning from each hybrid plant in the phytotron, fixed in Carnoy's solution [ethanol: acetic acid (3:1)] (Larter and Gustafson 1980; Li et al. 1995) overnight, transferred into fresh fixative solution every day until the solution became clear and stored in a freezer until use. The anthers were hydrolyzed in 1 N HCl at 60 °C for about 2 min and squashed gently in a drop of 10 % modified carbol fuchsin. A total of 100 PMCs were observed for each hybrid plant under the microscope.

Pollen viability

Pollen viability was measured by staining pollen grains from three young open flowers with 1 % acetocarmine. Approximately 600 pollen grains from anthers of three flowers were counted from each F_1 hybrid plant. Normal pollen grains were large, round and densely stained red and easily distinguished from small, non-stained dead pollen and immature microspore stage cells (Li et al. 1995).

Microspore isolation and regeneration

In a preliminary study, about 80 flower buds were collected from cuttings of 10 F_1 hybrids in the phytotron, placed on ice and brought to the laboratory where buds with length of 2–4 mm were selected and sterilized in 1 % hypochlorite for 15 min. The sterilized buds were transferred to a 100 μm sterile filter in the laminar flow and washed with 800 ml sterile water. After washing, the buds were transferred to a sterile beaker and 5 ml 1/2 B5-13 (half strength B5 Gamborg medium from Austratec TM plus 13 % sucrose in Millipore water, pH to 5.8). The microspores were released into the solution by gently squeezing buds using a glass rod. Then the solution with microspores were filtered through a 44 μm filter into a sterile 50 ml tube and adjusted to 30 ml with 1/2 B5-13 media. The microspores were centrifuged for 5 min at 1,200 rpm, the supernatant was discarded and resuspended in 30 ml fresh 1/2 B5-13 medium and centrifuged again for 5 min at 1,200 rpm. The supernatant was discarded again and the microspores were resuspended in 30 ml NLN-13 solution (NLN medium powder from Austratec plus 13 % sucrose in Millipore water, pH to 5.8).

Microspore suspensions were prepared following the method of Cousin and Nelson (2009) and Nelson et al. (2009), transferred to fresh NLN-13 medium and adjusted to a density of 4×10^5 mL^{-1}. The microspore suspension was divided into 10 mL lots, to which were added 100 μL 1 % activated charcoal and 0.5 % colchicine in Petri dishes. All the Petri dishes were sealed and wrapped with aluminium foil. Microspores were induced by a heat treatment at 32.5 °C for three days, after which 10 mL NLN-13 medium was added to each dish, and the plates were then placed in a culture room at 25 °C. Two weeks later, the plates were put on a shaker (60 rpm) for 1 week,

foil was removed, and they were placed in light (60 μmol m^{-2} s^{-1}) on a shaker for one more week. The dishes were transferred to 4 °C for storage and cold treatment to enhance embryo development, following the method of Zhang et al. (2006). Two weeks later, young embryos with good quality were transferred to 90 mm Petri dishes (25 embryos per dish) and counted.

Four F_1 hybrids were chosen for further manipulation of plantlets in culture as described by Takahira et al. (2011). Regenerated shoots with roots were transferred into soil in pots with a clear plastic cover to increase humidity for 1 week in the phytotron.

Flow cytometry analysis

Approximately 5 mg of fresh young leaf material was collected from microspore-derived (MD) plantlets in the phytotron, chopped by razor and prepared for flow cytometry following the conventional method, using lettuce as a control, as described by Nelson et al. (2009). The stained nuclei samples were analyzed using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) flow cytometer with a 488 nm laser. The genome size of samples was estimated through the use of a reference standard (lettuce) with known genome DNA content of 5.95 pg (Nelson et al. 2009). In addition, control *Brassica* samples were included, including allotetraploid *B. napus*. The absolute DNA amount of a sample was calculated based on the values of the G1 peak means: Sample 2C DNA content = [(sample G1 peak mean)/(standard G1 peak mean)] × [standard 2C DNA content (pg DNA)].

Sample data were acquired using BD FACSDiva V6.1.1 (BD Biosciences San Jose, CA, USA), which is the operating software on the FACSCanto II. Experimental data were analyzed and CV calculations performed using FlowJo V7.2.5 (Tree Star Inc., Ashland, OR, USA) flow cytometry analysis software. Samples were classified as hexaploid, triploid, mixed or other types of ploidy.

Statistical analysis

The data were analyzed using a statistical package, SPSS version 16.0 (SPSS, Chicago, IL, USA). The variation among F_1 hybrids in pollen viability, production of normal cells, four different abnormal cell types during meiosis, embryo production and also plant regeneration in microspore culture, was evaluated by analysis of variance (ANOVA) followed by Tukey's Honest Significant Differences test. All statistical analysis data had three repeats. Results were considered significant at $P \leq 0.05$.

Results

Ten hexaploid F_1 hybrids were selected for these studies based on diverse pedigree and stable and high chromosome numbers in parent lines, relatively high pollen viability and high seed yield of the F_1 hybrids in glasshouse, and high rates of embryogenesis, plantlet regeneration rate and chromosome doubling ability in microspore culture (Table 1). The grandparents of the hexaploid hybrids included 5 of the 6 *Brassica* species in U's triangle (U 1935) (Table 1), which improved the likelihood of finding high genetic diversity between parents in the A, B and C genomes.

Chromosome behavior and pollen viability in hexaploid F_1 hybrids

Chromosome number of parent plants used in crossing, and 10 hexaploid F_1 hybrid plants, were close to or precisely 54 (Table 1; Fig. 1). Pollen viability in 10 F_1 hybrids ranged from a low of 20.8 % in H02-4 to a high of 95.7 % in H04-6 (Table 1; Fig. 2).

Chromosome behavior during meiosis and microspore isolation from four hexaploid hybrids

We chose four hexaploid F_1 hybrids (H08-1, H11-2, H16-1 and H24-1) for further cytogenetic studies and microspore culture, based on the hexaploid status and genomic diversity of the parents, high chromosome number, relatively high pollen viability, high seed yield in the F_1 hybrids in the glasshouse, and preliminary microspore culture results (Table 1).

The four hexaploid F_1 hybrids were examined for chromosome pairing at diakinesis of metaphase I in PMCs. Five different kinds of PMCs were observed during meiosis I and II, which included normal cells, cells with lagging chromosomes, triads, polyads and cells with unequal division (Fig. 3). The percentage of cells with "normal" pairing was highest in F_1 hybrid H11-2 (70 %) and lowest in H16-1 (36 %) (Table 2). Several cells showed univalents or multivalents (Fig. 3), possibly as a result of homoeology between chromosomes in the A, B and C genomes. These abnormal chromosomes were not on the equatorial plate at metaphase I, or they moved towards opposite poles at anaphase I.

The four hexaploid F_1 hybrids showed a range of embryo generation ability and plantlet regeneration rate during microspore culture. The number of embryos per Petri dish (an average of 3 dishes per hybrid isolated on three different days) varied significantly between hybrids. The embryos with good quality were transferred into solid medium to generate plantlets. Hybrid H11-2 had the

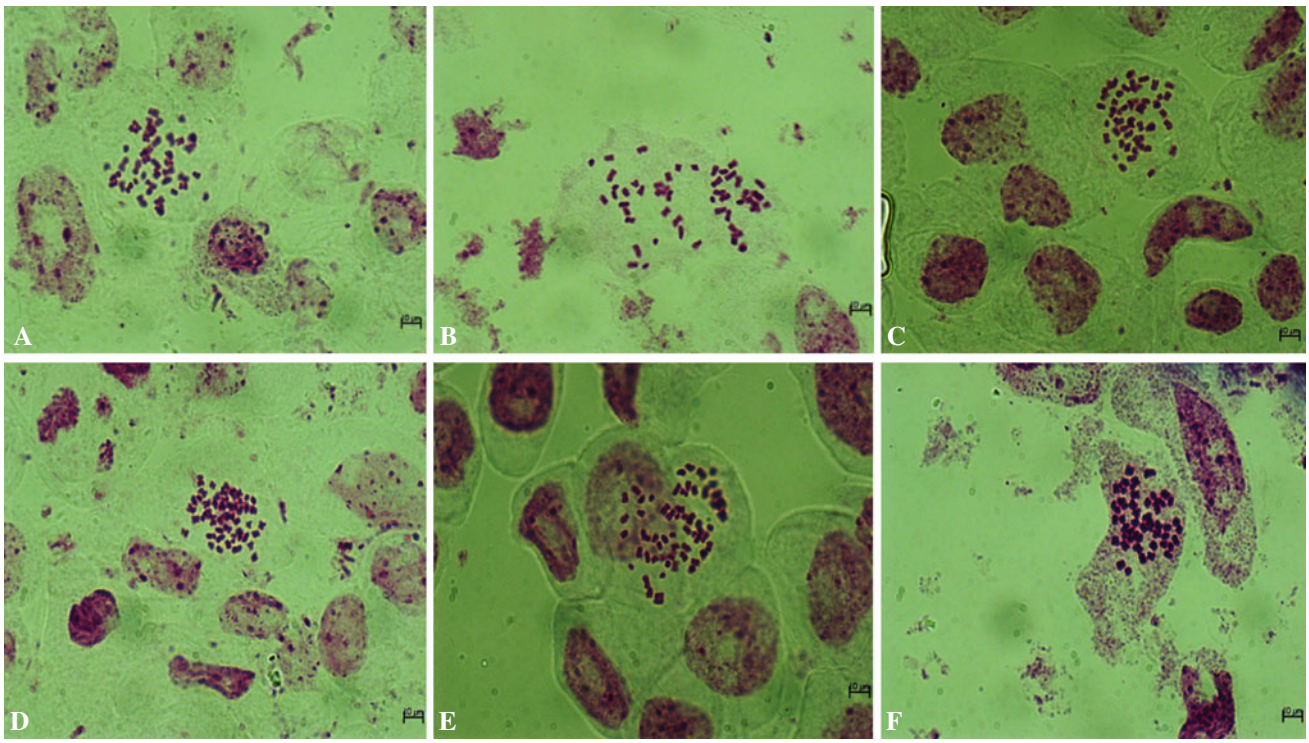

Fig. 1 Cytogenetic observations of chromosome numbers in somatic cells of young ovaries in 10 F_1 hybrid plants in the controlled environment room. **a** 45 chromosomes in H13-1, **b** 53 chromosomes in H01-6, **c** 54 chromosomes in H08-1, **d** H11-2, **e** H16-1 and **f** H24-1

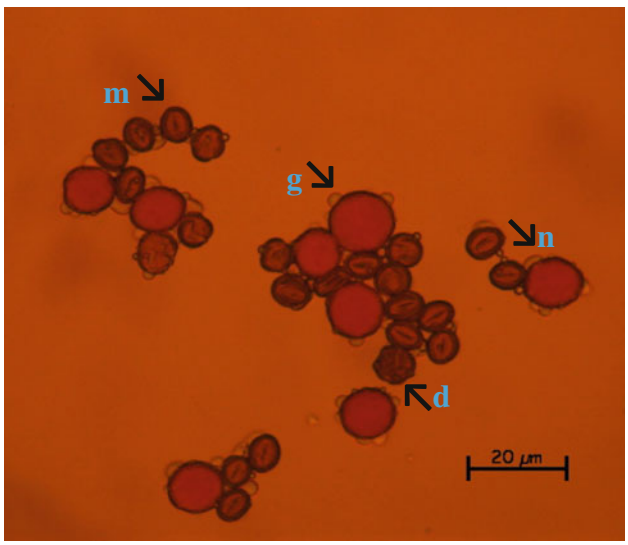

Fig. 2 Pollen viability of F_1 hybrids demonstrating normal pollen (n), giant pollen (g), microspore cell (m) and dead cell (d) in H16-1

highest number (394) of embryos per dish and also had the highest number of plantlets (693 plantlets) and plantlet regeneration rate (86.8 %) (Table 3). Hybrid H16-1 had 172 embryos per dish, the second highest number of plantlets (463 plantlets) and plantlet regeneration rate (72.7 %) (Table 3). Hybrid H08-1 had the second highest number (286) of embryos per dish, only 54 plantlets and plantlet regeneration rate (50.5 %) (Table 3). Hybrid H24-1 had 64 embryos per dish, only 56 plantlets and the lowest plantlet regeneration rate (11.2 %) (Table 3).

Ploidy level in microspore-derived plants using flow cytometry

The ploidy level of all MD plantlets from the four hexaploid F_1 hybrids was estimated from nuclear DNA content measured in flow cytometry (Nelson et al. 2009). The peak of nuclear DNA content (PI_DNA-A) by flow cytometry for *B. napus* was found to be at approximately 50,000 units, with and without lettuce, which had a peak of PI_DNA-A at approximately 125,000 units (Figs. 4a, b). The PI_DNA-A of hexaploid MD progeny and parents was about 75,000 units (Fig. 4c) and triploid MD progeny was about 37,500 units (Fig. 4d). Based on flow cytometry tests, MD plantlets in the four populations were categorized as hexaploid, triploid, mixed or "other" (unidentified) ploidy level (Table 4). In these experiments, DH progeny were hexaploid and haploid progeny were triploid, since the parents were hexaploid and the chromosome numbers of MD progenies were checked (Fig. 5). Hybrids H11-2 and H16-1 gave the largest DH populations for genetic mapping (370 and 241 DH progeny, respectively), whereas H08-1 and H24-1 each resulted in less than 25 DH progeny (Table 4).

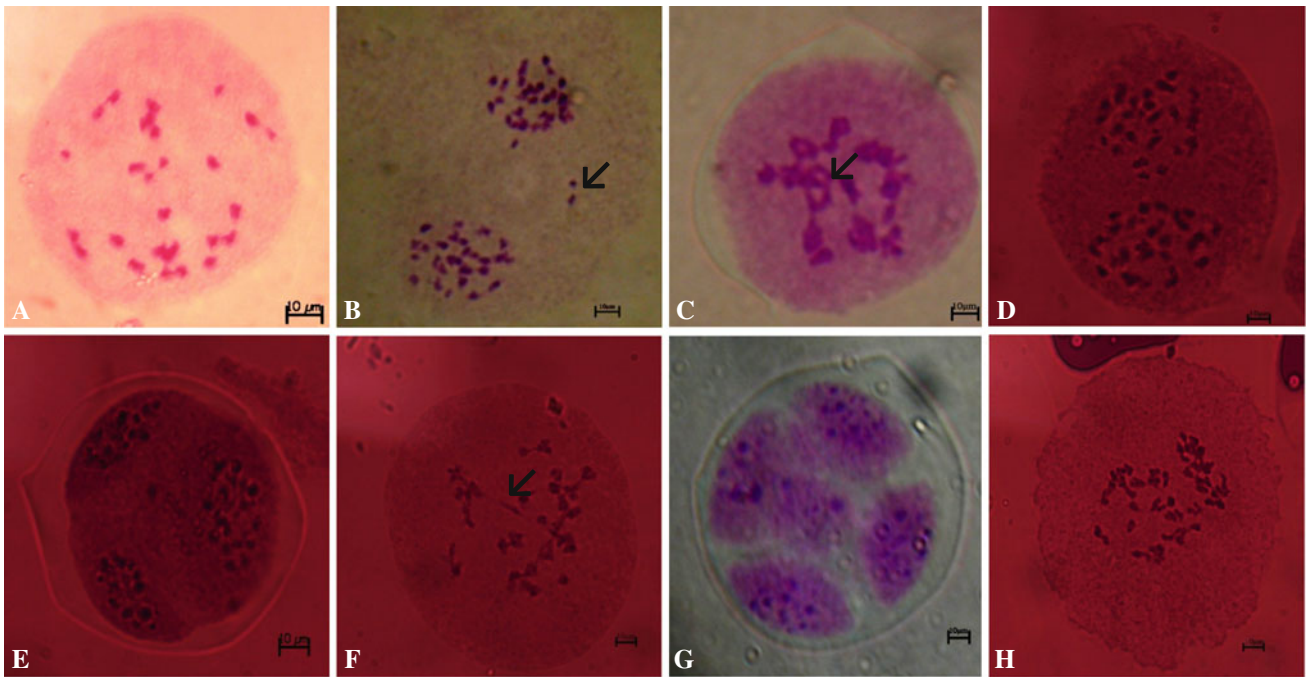

Fig. 3 Chromosome pairing in PMCs of four hexaploid F_1 hybrids. **a** Diakinesis PMCs with 27 II in H24-1. **b** PMC of H24-1 with a lagging bivalent at anaphase I. **c** Diakinesis PMC of H08-1 showing multivalents. **d** PMC of H24-1 showing normal segregation at anaphase I. **e** PMC of H11-2 showing triad at anaphase II. **f** PMC with a chromosome bridge. **g** PMC of H16-1 showing polyad. **h** PMC of H08-1 with unequal segregation at anaphase I

Table 2 Chromosome behaviour in anaphase I and II during meiosis in pollen mother cells of four hexaploid *Brassica* F_1 hybrids as observed in 100 pollen mother cells per plant

Hybrid code	Plant no.	27 pairs chromosomes[a]	Cells with lagging chromosomes	Cells with triads	Cells with sporads (>3 cells)	Cells with unequal cell division
H16-1	1	33	51	16	0	0
	2	41	58	0	1	0
	3	35	65	0	0	0
	Mean	36 c	58 a	5 a	1 a	0 a
H08-1	1	63	34	3	0	0
	2	65	32	2	0	0
	3	71	29	0	0	0
	Mean	66 ab	32 bc	2 a	0 a	0 a
H11-2	1	69	19	9	1	2
	2	70	22	7	0	1
	3	72	27	1	0	0
	Mean	70 a	23 c	5 a	1 a	1 a
H24-1	1	68	32	0	0	0
	2	58	41	1	0	0
	3	54	42	4	0	0
	Mean	60 b	38 b	2 a	0 a	0 a

Three plants per hybrid F_1 were selected for chromosome pairing observation. In "normal" cells, all chromosomes were in pairs (27 pairs) at diakinesis during anaphase I. "Abnormal" cells had some lagging chromosomes, triads, sporads (>3 cells) and unequal cell division during anaphase I and II

[a] Means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by Tukey's Honest Significant Differences test

DH progenies of hybrids selected as candidates for mapping population

The hexaploid hybrid H11-2 showed the highest "normal" gamete production, embryo generation ability and plantlet regeneration rate (Table 3), the highest doubling frequency (Table 4) and also formed the highest number of hexaploid DH progeny (Table 4). H16-1 produced the second highest number of hexaploid DH progeny and also showed lower embryo generation ability, the second highest number of

Table 3 Number of microspore embryos per Petri dish and plantlet regeneration rate of four hexaploid F_1 hybrids

Hybrid code	Embryo production	Plantlet regeneration rate			
	(number per Petri dish[a])	Normal plantlet	Dead plantlet	Callus	Total number of embryos[b]
H08-1	286 b	54 (50.5 %)	48	5	107
H11-2	394 a	693 (86.8 %)	92	13	798
H16-1	175 c	436 (72.7 %)	136	28	600
H24-1	64 d	56 (11.2 %)	357	87	500

[a] Means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by Tukey's Honest Significant Differences test

[b] The total number of embryos with good quality derived from microspore culture were selected and transferred into solid MS medium to regenerate shoots

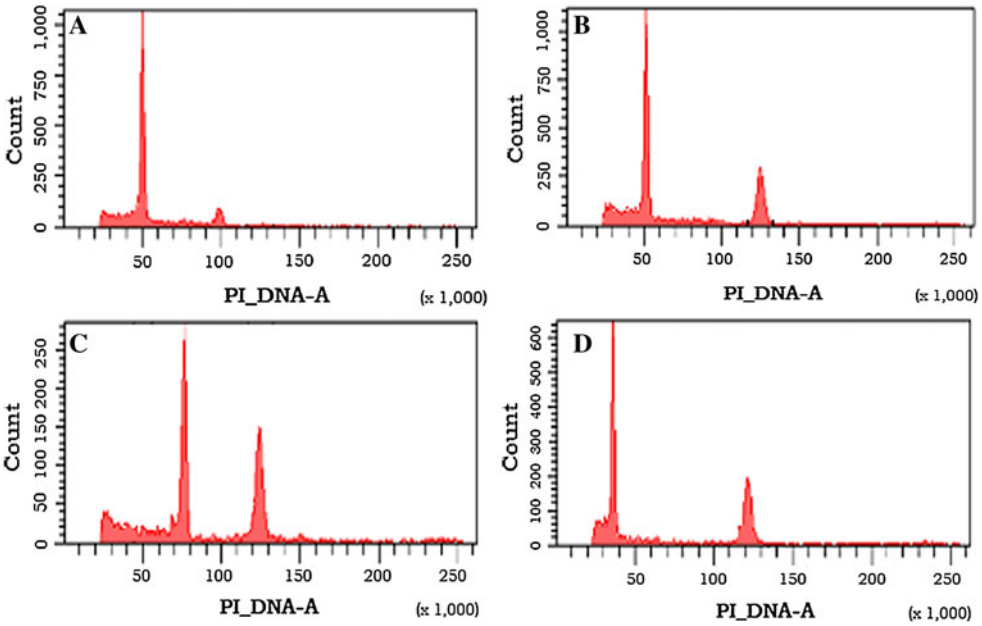

Fig. 4 Examples of flow cytometry results. **a** *Brassica napus* (the peak of nuclear DNA content is 50,000 units), **b** *B. napus* (the peak of nuclear DNA content is 50,000 units) + lettuce (the peak of nuclear DNA content is 125,000 units), **c** microspore-derived (MD) hexaploid plant H16-1-002 + lettuce (the peak of nuclear DNA content is 75,000 units), and **d** MD triploid plant H16-1-011 + lettuce (the peak of nuclear DNA content is 37,500 units)

Table 4 Classification of ploidy level in microspore-derived plants by flow cytometry based on DNA content of controls with known DNA content. Plants were classified as hexaploid (6*x*), triploid (3*x*), mixed, or unknown, and doubling frequency (%) is the proportion of hexaploids in the total

Hybrid code	Hexaploid	Triploid	Mixed	Unknown	Total	Doubling frequency (%)
H08-1	23	16	4	11	54	42.6
H11-2	370	298	6	19	693	53.4
H16-1	241	185	3	7	436	55.3
H24-1	21	19	2	14	56	37.5

plantlet regeneration rate but lowest "normal" gamete production. H08-1 had the lower number of hexaploid DH progeny, the second highest embryo generation ability, lower plantlet regeneration rate and lower doubling frequency. H24-1 had the lowest embryo production per dish and plantlet regeneration rate (Table 3), lowest doubling frequency (Table 4), and lowest number of hexaploid DH progeny formed (Table 4). High correlation was found between embryo number per Petri dish and plantlet regeneration rate ($r^2 = 0.82$), and between embryo number per Petri dish and doubling frequency ($r^2 = 0.91$) for the four hybrids used in microspore culture (H08-1, H11-2, H16-1, and H24-1). Based on these findings, DH progenies of two hybrids H11-2 and H16-1 were proposed as candidates for allohexaploid mapping populations.

Discussion

The most common method of producing artificial *Brassica* allohexaploids is crossing an allotetraploid and a diploid species with a complementary genome, and then doubling

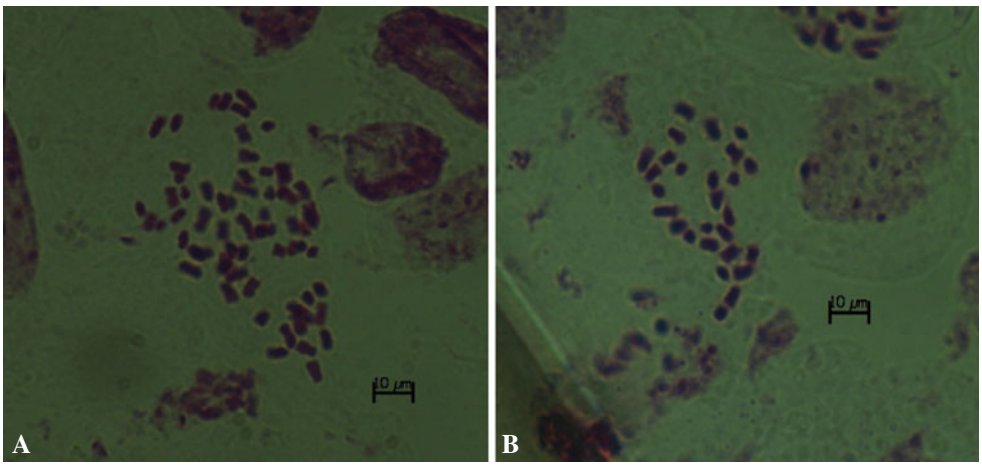

Fig. 5 Chromosome numbers in somatic cells of young ovaries in microspore culture-derived plants in glasshouse. **a** 54 chromosomes in H16-1-002 (6*x* DNA content by flow cytometry), and **b** 27 chromosomes in H16-1-008 (3*x* DNA content by flow cytometry)

the chromosome complement of the F_1 (a sterile triploid) through the use of colchicine (Li et al. 2005a, b; Tian et al. 2010; Ge et al. 2009; Pradhan et al. 2010; Mason et al. 2012). Many polyploid plants have been produced via chromosome doubling technology including *Brassica* (Meng et al. 1998; Li et al. 2004), potato (Johnstone 1939; Saisingtong et al. 1996) and tobacco (Takashima et al. 1995). Synthetic hexaploid wheat has been generated by crossing tetraploid durum wheat (2n = 4*x* = 28, AABB) with diploid *Aegilops tasschii* Coss (2n = 2*x* = 14, DD) to generate genetic diversity for modern wheat breeding (Dreisigacker et al. 2008). The hexaploid triticale (AABBRR) is a successful synthetic allopolyploid derived through crossing tetraploid wheat (AABB) and rye (RR) (Ammar et al. 2004). The stability of triticale was highly improved after the lines from different genetic backgrounds were intercrossed and selected for genomic stability. Triticale has been widely used in agricultural production (Comai 2000).

In our research, a number of crosses were made between allohexaploid *Brassica* parents from diverse genetic backgrounds. All the lines used as parents in this study had close to the expected hexaploid number of 54 chromosomes and around 50 % normal pollen grains, and were derived from various genetic backgrounds including almost all the species of the *Brassica* U triangle (Table 1). Four hexaploid hybrid plants were chosen for microspore culture to establish future DH mapping populations of hexaploid *Brassica*.

Chromosome stability is the major factor for the establishment and persistence of polyploidy in plants. Many artificial allopolyploids, such as *Triticum*, *Gossypium* and *Arabidopsis*, show homologous chromosome pairing and stable chromosome number during meiosis after several generations of selfing (Comai 2000; Liu et al. 2001; Zhang et al. 2004). New synthetic allohexaploid wheat hybrids inherited meiotic irregularities which were progenitor-dependent (Mestiri et al. 2010). We used a range of sources of allohexaploid *Brassica*, which were relatively genetically stable after several generations of selfing (Ge et al. 2009), as parents in our crossing experiments.

Cytological observation of chromosome behavior during meiosis in allohexaploid parents demonstrated abnormal chromosome pairing and segregation, such as laggards, triads, polyads, unequal cell division, chromosome bridges and chromosome loss. The appearance of a chromosome bridge and lagging chromosomes indicated that pairing of homoeologous chromosomes in different genomes may lead to chromosome rearrangements such as inversions or exchange (Busso et al. 1987). Meng et al. (1998) and Rahman (2001) produced a pentaploid line (AABCC) through intercrossing the artificial hexaploid (AABBCC) and *B. napus* (AACC). They found some *B. napus* (2n = 38) from the selfing progenies of this pentaploid line. This showed that the B genome chromosomes are lost during selfing and rarely pair with A and C genome chromosomes (Rahman 2001).

Recently, Tian et al. (2010) proved by in situ hybridization that the lagging chromosomes during meiosis of a *Brassica* hybrid pentaploid were mostly either B genome or A/C pairs. Nearly 50 % of the PMCs had trivalents and quadrivalents, which revealed partial homology among the A, B and C genomes. The bivalents most likely belonged to the B genome. In our research, we observed abnormal chromosome behavior during meiosis in hexaploid *Brassica* parents and DH progeny that might be due to pairing between homologous and non-homologous regions.

It is possible that, given the occurrence of giant pollen cells (Fig. 3), some of the 6*x* progeny were products of unreduced gametes rather than colchicine-induced doubled haploids, as was found in microspore-derived progeny of

an unbalanced ABCC hybrid reported by Nelson et al. (2009). Molecular marker characterisation of the 6*x* progeny would distinguish between DH (all homozygous loci) and unreduced gametes (combination of heterozygous and homozygous loci).

F_1 hybrids H11-2 and H08-1 had high embryo production, as well as high "normal" gamete production. The parents of both hybrids included an allohexaploid parent plant derived from *B. rapa* × *B. carinata* and a parent plant derived from (*B. juncea* × *B. napus*) × *B. carinata* (Table 1). The F_1 hybrids H24-1 and H16-1 had lower embryo production. The parents of these two hybrids included an allohexaploid parent plant derived from *B. rapa* × *B. carinata* and a parent plant derived from *B. napus* × *B. nigra* (Table 1). It seems that the F_1 hybrids H11-2 and H08-1 from *B. rapa* × *B. carinata* and (*B. juncea* × *B. napus*) × *B. carinata* were much easier to obtain embryos than the other cross. *B. nigra* in the parentage may have reduced the ability of H24-1 and H16-1 to form embryos in microspore culture, as this species has been recalcitrant in microspore culture (Govil et al. 1986; Hetz and Schieder 1989). The more successful F_1 hybrids H11-2 and H08-1 included parentage from all three allotetraploid *Brassica* species plus diploid *B. rapa*. Less successful F_1 hybrids H24-1 and H16-1 were derived from two allotetraploids and two diploid *Brassica* species.

We found two F_1 hybrids (H11-2 and H16-1) that generated several hundred allohexaploid DH progenies as shown by flow cytometry. Both of these hybrids have different sources of A, B and C genomes from their grandparents, and are therefore genetically diverse and ideal for genetic mapping of various traits including chromosome pairing stability in a new *Brassica* hexaploid species.

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