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ORIGINAL PAPER

Synthesis of a *Brassica* trigenomic allohexaploid (*B. carinata* × *B. rapa*) de novo and its stability in subsequent generations

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Abstract Allopolyploidy plays an important role in plant evolution and confers obvious advantages on crop growth and breeding compared to low ploidy levels. The present investigation was aimed at synthesising the first known chromosomally stable hexaploid *Brassica* with the genome constitution AABBCC. More than 2,000 putative hexaploid plants were obtained through large-scale hybridisation from various combinations of crosses between different cultivars of *Brassica carinata* (BBCC) and *B. rapa* (AA). The majority of plants after two generations of selfing within selected hexaploid plants (H_2) were aneuploid, and only 80 plants (4.6%) had the expected hexaploid chromosome number ($2n = 54$). The hexaploid ratio increased to an average of 23.0 and 26.3% in the H_3 and H_4 generations, respectively, and was accompanied by an increase in pollen fertility. The appearance of aneuploid plants in each generation could be detected having various chromosomal abnormalities at meiosis. The frequency of hexaploid plants varied significantly among different cultivar combinations, from 0 to 56% in the H_4 generation, and it showed a positive correlation with pollen fertility. The frequency of SSR allelic fragments lost or novel alleles gained was significantly lower in H_4 than in H_2 and H_3, which reflects increasing genome stability in H_4. The A and C genomes were significantly less stable than the B genome, which may mainly result from frequent homoeologous pairing and rearrangements between the A and C genomes. Methods to establish a stable hexaploid *Brassica* crop by intercrossing these lines followed by intensive selection are also discussed.

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Introduction

Polyploidisation is one of the major forces in plant evolution and speciation (Soltis and Soltis 1995; Ramsey and Schemske 1998; Bennett 2004). It is estimated that approximately 70% of angiosperms have experienced one or more chromosome doubling events during their evolutionary history (Masterson 1994). In the genus *Brassica*, the U-triangle (U 1935) represents a classical evolutionary process, in which the three diploid species viz. *B. rapa* (AA, $2n = 20$), *B. nigra* (BB, $2n = 16$) and *B. oleracea* (CC, $2n = 18$) originated three tetraploids species: *B. carinata* (BBCC, $2n = 34$), *B. juncea* (AABB, $2n = 36$) and *B. napus* (AACC, $2n = 38$) by pairwise hybridisation and subsequent chromosome doubling in the natural environment. Polyploidy in *Brassica* is confined to tetraploidy, because no higher polyploid species of *Brassica* (e.g., AABBCC) exist in nature, although several other crop

species, such as hexaploid wheat (*Triticum aestivum*, genome AABBDD) and hexaploid oat (*A. sativa* L. and *A. byzantina*, genomes AACCDD), display a higher level of ploidy.

There is a long history of trigenomic hexaploid *brassica* synthesis, which is derived following hybridisation between the tetraploid and diploid species. In earlier years, these were attempted primarily for academic purposes. The allohexaploid *B. carinata* × *B. rapa* has been synthesised widely in our previous investigations, and used as a bridge to create novel *B. napus* genotypes exhibiting useful characters such as yellow seeds and displaying strong intersubgenomic heterosis (Meng et al. 1998; Li et al. 2004, 2006; Zou et al. 2010). Using somatic cell hybridisation in several investigations, the hexaploids originated following somatic hybridisations and were used as a "bridge". These include *B. juncea* + *B. oleracea* for transferring '*tour*' cytoplasmic male sterility into the digenomic *Brassica* species (Arumugam et al. 1996) and *B. napus* + *B. nigra* to introgress genes for resistance to diseases such as *Phoma lingam* and *Plasmodiophora brassicae* from *B. nigra* to rapeseed (Sacristán et al. 1989; Gerdemann et al. 1994; Sjödin and Glimelius 1998). However, because of chromosomal instability and very poor seed fertility, these hexaploids could not be established. Normally, genomic instability in newly synthesised polyploids is an important challenge for the survival of neopolylploids (Comai et al. 2000), which would significantly affect successful construction of the *Brassica* hexaploid as well.

The present investigation was aimed at establishing a new species of *Brassica* hexaploid. We developed a large population of hexaploids by crossing various accessions of *B. carinata* and *B. rapa* cultivars and investigated the chromosomal stability, fertility and genomic stability of the hexaploids extensively across various generations.

Materials and methods

Plant materials and field experiments

According to the crossing results between 29 accessions of *B. rapa* and 107 accessions of *B. carinata* in 2004 (Jiang et al. 2007), six accessions of *B. carinata* (Supplementary Table 1) with a higher than average degree of interspecific crossability to *B. rapa* were combined with 214 accessions of *B. rapa* (Supplementary Table 2) to generate more *Brassica* hexaploid plants. When the parental plants flowered in March 2005, pollen from each *B. rapa* accession was used to cross approximately 70 randomly chosen buds from the six *B. carinata* accessions. All putative triploid seedlings obtained were treated with 0.1% (w/v) colchicine, and the colchicine-treated generation was designated as H_1 (the first generation of hexaploid plants). The subsequent generations of selfing were in turn referred to as H_2, H_3, H_4 and so on. All materials used were planted in the field station of Huazhong Agricultural University, Wuhan, China. Each accession was planted in three rows, with eight plants per row; all rows were 2.0 m long, with 25.0 cm between rows. Seed setting was investigated in H_3 and H_4 of each combination using 30 siliques from each of three randomly selected individual hexaploid plants.

Cytological methods

Ovaries from young flower buds of hexaploid progeny plants were used for chromosome counting. The samples were treated with 2 mM 8-hydroxyquinoline for 4–5 h at room temperature, and then fixed in Carnoy's solution (95% ethanol:glacial acetic acid = 3:1). Young anthers containing pollen mother cells (PMC) were excised from fixed flower buds in Carnoy's solution for cytogenetic analysis according to the modified carbol fuchsin method (Li et al. 2001). Pollen fertility was determined as the ratio between the number of pollen grains that were stained with 1% acetocarmine and the total number of pollen grains; each sample comprised at least 300 pollen grains.

SSR analysis

Genomic DNA was extracted from young leaves of three randomly selected individual hexaploid plants for each early hexaploid generation (H_2 to H_4) of each combination (C03, C04, C05, C09, C11, C13, C14, C15), using a modified cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle 1990). SSR primers were used to analyse the genome stability in early generations of *Brassica* hexaploids. A/C genome-specific SSR markers were amplified from 118 primers distributed across the A and C genomes of *B. napus*, according to information from the TN DH genetic map (Qiu et al. 2006; Long et al. 2007). The B genome-specific markers were amplified from 11 primers that were selected from the Celera AgGen *Brassica* Consortium and JIC (http://www.brassica.bbsrc.ac.uk/BrassicaDB/), the National Institute of Agricultural Biotechnology of Korea and Agriculture and Agri-Food Canada (http://www.brassica.agr.gc.ca/index_e.shtml) (Supplementary Table 3). The genomic specificity of the markers was verified by comparison with SSR bands amplified from three control cultivars: *B. rapa* cv. WulitianYC, *B. nigra* cv. Koch 'Giebra' and *B. oleracea* var. alboglabra Bailey No. 4003. The SSR protocol was primarily followed Lowe et al. (2004).

Data analysis

Chromosomal stability in different combinations or generations of *Brassica* hexaploids was evaluated by the

frequency of hexaploid plants in all the sampled plants. SAS statistical package (1999) was used for multiple range tests, correlation coefficients and two-way analysis of variance on pollen fertility, seeds per pod and genome stability. In addition, the distribution of chromosome numbers in the three generations of hexaploids and the variation in pollen fertility and seeds per pod were analysed with the software package SPSS 10.0 (1999).

Results

Development of a *Brassica* hexaploid population by crossing various accessions of *B. carinata* and *B. rapa* cultivars

To introgress the wide variability available in *B. rapa* into the hexaploid population, 214 accessions of *B. rapa* of various origins were used to pollinate *B. carinata* (Table 1, Supplementary Table 2). The hybrid seeds had a low germination rate (23%), and some matromorphs (8.9%) were identified after morphological screening followed by chromosome counting and marker identification. After treatment with colchicine to induce allopolyploidy, all H_1 (the first hexaploid generation) plants were grown in the field. Chromosome doubling occurred in approximately 23% of hybrid plants and nearly all plants had at least one branch in which cells contained doubled chromosomes ($2n = 54$; Fig. 1a–d). The trigenomic hexaploids could easily be distinguished from their parents morphologically, because these plants were more vigorous and produced fewer seeds than their parents upon self-pollination. More than 2,000 H_1 plants were obtained from two experiments involving 457 different combinations of *B. carinata* and *B. rapa* cultivars. Flowers of the H_1 plants produced abundant pollen and were bagged to obtain self-pollinated seeds. The seed set in H_1 plants varied greatly. However, seeds were obtained from only 822 plants which had high seed setting ability (at least 50 seeds/plant) and were derived from 411 *B. carinata*/*B. rapa* combinations. This group included all H_1 plants with the expected 54 chromosomes and was used to produce the next generation.

Table 1 General information on the synthesis of *Brassica* hexaploids

	2004–2005 season	2003–2004 season (Jiang et al. 2007)	Total
Accessions of *B. carinata*	6	110	116
Accessions of *B. rapa*	214	29	243
Buds pollinated	14,000	40,000	54,000
Hybrid seeds obtained	19,000	13,000	32,000
Triploid plants obtained	4,500	5,100	9,500
No. of putative hexaploid plants	1,030 (265)	1,190 (192)	2,220 (457)

The number in brackets corresponds to the number of combinations of cultivars of *B. rapa* or *B. carinata* yielded hexaploid plants

Variation in chromosome number in the H_2 generation of the hexaploid

In the second hexaploid generation (H_2), more than 10,000 seedlings were grown in the field, but only 6,169 plants grew to maturity. The majority of the plants had very low fertility with very poor seed set, which indicated a severely unbalanced chromosomal constitution. On the other hand, a very small number of plants were fully fertile but morphologically resembled different *Brassica* species, i.e., *B. carinata* (36 plants), *B. napus* type (19), *B. rapa* type (15) and *B. juncea* type (7). Eventually, 28% (1,753) of the 6,169 H_2 plants with higher seed setting ability (at least 50 seeds/plant) were selected as candidates for chromosome counting. Only a small number of plants (80) contained the expected 54 chromosomes; in other plants, the chromosome number varied greatly, from 26 to 52 with 44 being the most common (Fig. 2). In summary, the H_2 generation contained three classes of plants: hexaploid (4.6%), aneuploid (91.0%) and lower ploidy euploid (4.4%). All hexaploid H_2 plants were morphologically similar to their H_1 parents and produced seeds normally.

The 80 hexaploid H_2 plants were derived from 35 cross combinations that involved 25 cultivars of *B. carinata* and 22 of *B. rapa* (Table 2). More than 40% of the H_2 hexaploid plants were derived from three combinations viz. C21, C28 and C15 (Table 2). Two crossable accessions of *B. carinata*, CGN03995 and CGN03949, yielded eleven and eight H_2 hexapoid plants with *B. rapa* cultivars, respectively. On the other hand, the cross *B. rapa* cv. WulitianYC × *B. carinata* cv. CGN03983 gave the highest number of H_2 hexaploid plants from a single cross combination, and also yielded one H_2 hexaploid plant with the crossable cultivar CGN03949. Fifteen H_2 hexaploid plants with various degrees of chromosomal stability were chosen from combinations C01–C15 to analyse for inheritance of chromosomal stability and fertility.

Chromosomal stability and fertility in the early successive generations

Plants (24) were grown for each of the 15 H_3 families, which were derived from the H_2 plants described above, and this resulted in a total of 359 H_3 plants in the field. Fertility in the H_3 population was much better than in the

Fig. 1 Production of AABBCC hexaploids. **a** Field view of ABC hybrid and the parents, **b** fertile hexaploid flowers with pollen (*top left*), **c**, **d** seed bearing branches of hexaploid plants, **e** comparison of seed set in hexaploid and three alloploid *Brassica* species

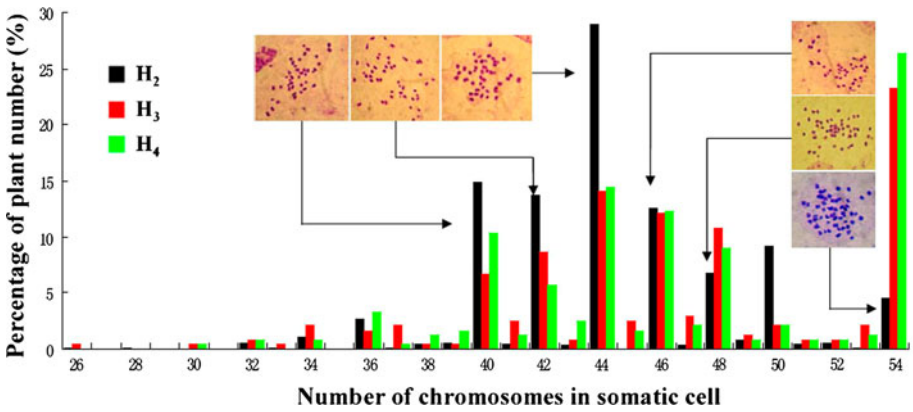

Fig. 2 Variations in chromosome number in three generations of hexaploid, six images show different somatic chromosome numbers, i.e. 40, 42, 44, 46, 48 and 54 in H_2 generation

H_2 generation, with only a quarter of the plants being poorly fertile. Plants with higher seed setting ability (271 plants with at least 50 seeds/plant) were subjected to chromosome counting, and 63 were shown to be hexaploid, a level that was 5- to 18-times higher than that of the H_2 generation (Fig. 2). It was observed that the frequency of hexaploid plants varied a great deal among the H_3 families; it ranged from 6 to 44% with an average of 23% (Fig. 3).

One hexaploid plant with good fertility was selected from each H_3 family to yield an H_4 population, which contained the same number of plants as the H_3 generation. The frequency of plants with poor seed setting ability decreased to 20%, and 280 H_4 plants exhibiting good fertility were selected for determination of chromosome number. The results revealed that 26.3% of H_4 plants were hexaploid, which was 3% higher than for the H_3 generation. The variation between H_4 families with respect to the frequency of hexaploid plants also increased compared to that for the H_3 generation; it ranged from 0% in the C04 family to 56.0% in the C15 family. Correlation analysis showed that there was a significant positive correlation for the frequency of hexaploid plants between the H_3 and H_4 generations of 15 hexaploid families (Table 3).

Pollen fertility and seed number per pod in the hexaploid plants increased significantly in the H_3 generation and increased in the H_4 generation. There was no significant difference in seed setting between *B. carinata* and the H_4 hexaploids (Supplementary Table 4; Fig. 4). The best

Table 2 Pedigree of hexaploid plants identified in the H_2 generation

Combination code	Cultivars crossed (*B. carinata* × *B. rapa*)	Number of hexaploid plants	Combination code	Cultivars crossed (*B. carinata* × *B. rapa*)	Number of hexaploid plants
C01	03934[a] × WulitianYC	1	C20	03975 × Denglongzhong	3
C02	03940 × Maverick	1	C21	03983 × WulitianYC	17
C03	03941 × Chuanbaicai	1	C22	03984 × FenyangYC	1
C04	03941 × ZhenxiongdaYC	1	C23	03984 × Yangyou2hao	1
C05	03943 × 3907	3	C24	03986 × TianmenYCbai	1
C06	03949 × WulitianYC	1	C25	03988 × Qixingjian	1
C07	03949 × DuyunYC	1	C26	03989 × DongkoutianYC	2
C08	03949 × TianmendayeYC	1	C27	03994 × FenyangYC	1
C09	03949 × RuidianSwedish	1	C28	03995 × Baijian 13	11
C10	03949 × SOLO	1	C29	03995 × ShangdangYC	1
C11	03949 × KaiyangtianYC	1	C30	03996 × TianmendayeYC	4
C12	03949 × DaqiaoYC	1	C31	04000 × TianmendayeYC	1
C13	03949 × Ankangzhong	1	C32	04003 × 3907	1
C14	03953 × DongkoutianYC	1	C33	04005 × XinghuaYC	1
C15	03955 × BaiguotianYC	6	C34	04006 × XinghuaYC	3
C16	03963 × Denglongzhong	1	C35	04030 × Yangyou2hao	2
C17	03964 × Maverick	1	Total:		
C18	03965 × TianmenYCbai	4	Combination	*B. carinata* / *B. rapa*	Hexaploid plants
C19	03968 × Maverick	1	35	25 / 22	80

[a] Three characters (CGN) in the accession codes of *B. carinata* were omitted

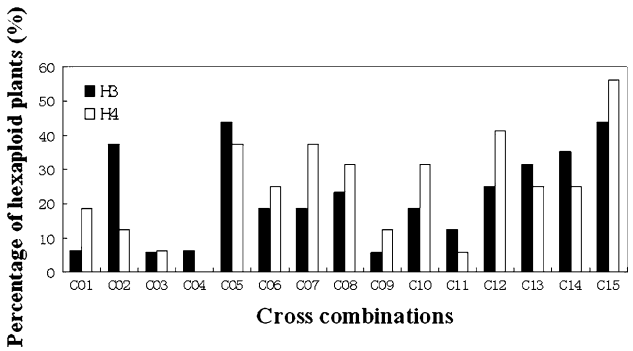

Fig. 3 Frequency of hexaploid plants in 15 families investigated over two generations. Chromosome number in 17–19 plants for each family/generation was analysed

H_4 hexaploid families had pollen fertility equivalent to *B. rapa*, and their seed setting ability was higher than that of *B. carinata*: the C05 family yielded 15.6 seeds per pod, which was equal to that of the higher seed setting parent *B. rapa* (Fig. 4). Pollen fertility correlated positively with the frequency of hexaploid plants, especially for pollen fertility in the H_3 generation and the frequency of hexaploid plants in the H_4 generation. No obvious correlation was found for seed setting.

Table 3 Correlation coefficients for the frequency of hexaploid plants and pollen fertility in 15 hexaploid families

	1	2	3	4
1. Pollen fertility in H_3	1.00			
2. Pollen fertility in H_4	0.00	1.00		
3. Frequency of hexaploid plants in H_3	0.63*	0.20	1.00	
4. Frequency of hexaploid plants in H_4	0.65*	0.14	0.61*	1.00

* Designates significance at $p < 0.05$

The chromosomal instability and reduction in fertility of the hexaploid progeny may result from many factors, the most important of which might be irregular chromosome pairing and abnormal segregation. Therefore, further cytological investigation was carried out in the H_3 family of C15. The frequency of bivalents was 23.42 per PMC which was higher than the value of 22.30 per PMC in H_2 but lower than the expected frequency of 27 bivalents per PMC. Correspondingly, a high frequency of univalents, trivalents and quadrivalents was found per PMC (Table 4). In addition, a high frequency of abnormalities related to chromosome segregation was found (Fig. 5a–d). For example, precocious chromosome migration at both poles and laggards occurred in more than half of the observed cells at metaphase and anaphase of meiosis.

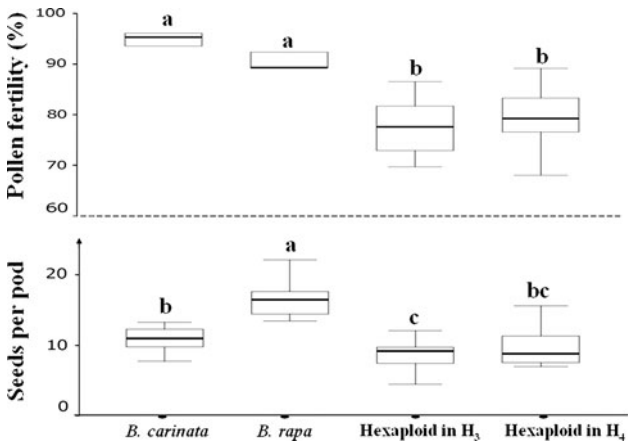

Fig. 4 Comparison of fertility characteristics among hexaploid plants in the H_3 and H_4 generations and their parental species. The *upper* and *lower whiskers* of the *boxplots* define the range of variation in fertility for each parent and hexaploid generation, whereas the *boxes* show the interquartile range (25th–75th percentiles) and the *thick horizontal lines* within *boxes* represent the median values or second quartiles. *Boxes with the same letters* over them are not significantly different among *B. carinata*, *B. rapa*, hexaploids in H_3 and hexaploids in H_4, according to Duncan's multiple range test ($p = 0.05$)

Genome stability of hexaploid plants in the early generations evaluated with molecular markers

The genome stability of eight combinations was evaluated in the early successive generations (H_2–H_4) with 1,700 molecular markers. Compared with their original triploids and parental species (*B. carinata* and *B. rapa*), the genomes of hexaploid plants displayed instability at very early generations (H_2 and H_3) with 2.77% of novel alleles appearing and 2.76% of parental alleles disappearing. However, the stability increased significantly in the H_4 generation, showing a 1.64-fold increase in the index of stability (Table 5). There was no significant difference ($p = 0.098$) in genome stability between the two sets of combinations that showing a higher (C05, C13, C14, C15) and lower (C03, C04, C09, C11) frequency of hexaploid plants across H_3 and H_4 generations (Fig. 3; Supplementary Table 4), which implied that chromosome stability had a weak effect on genome stability. Moreover, there was a positive but not significant relationship between genome stability and the seed setting ($r = 0.349$, $p = 0.185$) and pollen fertility ($r = 0.296$, $p = 0.266$), indicating that consecutively increasing genome stability might improve the fertility of newly synthesised *Brassica* hexaploids. The molecular markers that had been shown to be genome-specific for A (505), B (96) and C (162) genomes of *Brassica* were used to evaluate stability differences in various genomes in the early hexaploid generations. The A and C genomes were less stable than the B genome (Table 5).

Discussion

Synthetic hexaploids following the cross *B. carinata* × *B. rapa* or reciprocals have been obtained since 1942, as reported by Howard (1942; *B. rapa* ssp. *chinensis* × *B. carinata*), Mizushima (1950; *B. carinata* × *B. rapa* ssp. *pekinensis*), Olsson (1963; *B. carinata* × *B. campestris*), Iwasa (1964; *B. carinata* × *B. rapa* ssp. *pekinensis*) and Takeda (1967; *B. carinata* × *B. campestris*). In all these investigations, synthesis was attempted at either the diploid or tetraploid level, and a reasonable number of hybrids were produced (for details see Prakash and Hinata 1980). In the present investigation, a large variation was observed among parental genotypes, in terms of both their cross-ability and ability to produce stable hexaploid progenies. Use of *B. carinata* accessions with better crossability enabled us to obtain a large number of hexaploid plants efficiently from different combinations of *B. carinata* and *B. rapa* cultivars. This provided plenty of resources for us to identify genetically stable hexaploid plants by analysing chromosomal stability and fertility from early to late generations.

Chromosomal stability is the key factor for the establishment and persistence of polyploidy in plants. It is not a serious problem in resynthesised polyploids such as *Triticum*, *Gossypium* and *Arabidopsis*, in which chromosomes usually pair well during meiosis and the chromosome number tends to be stable after few generations

Table 4 Statistics for chromosome pairing configurations and chromosomal abnormalities

Generation	Chromosome paring configurations at diakinesis					Chromosomal abnormality[a]	
	No. of observed cells	Univalents	Bivalents	Trivalents	Quadrivalents	No. of observed cells	Ratio of abnormal cells (%)
H_2	50	1.44 (0–3)[b]	22.30 (19–27)	1.80 (0–3)	0.64 (0–2)	447	52.5
H_3	42	1.18 (0–3)	23.42 (19–27)	1.39 (0–3)	0.45 (0–1)	542	52.2

[a] The chromosomal abnormality is mainly related to laggards (anaphase I and anaphase II) and precocious chromosome migration (metaphase I and metaphase II) for meiosis in H_2 and H_3 generations

[b] Variation of configuration per pollen mother cell (PMC)

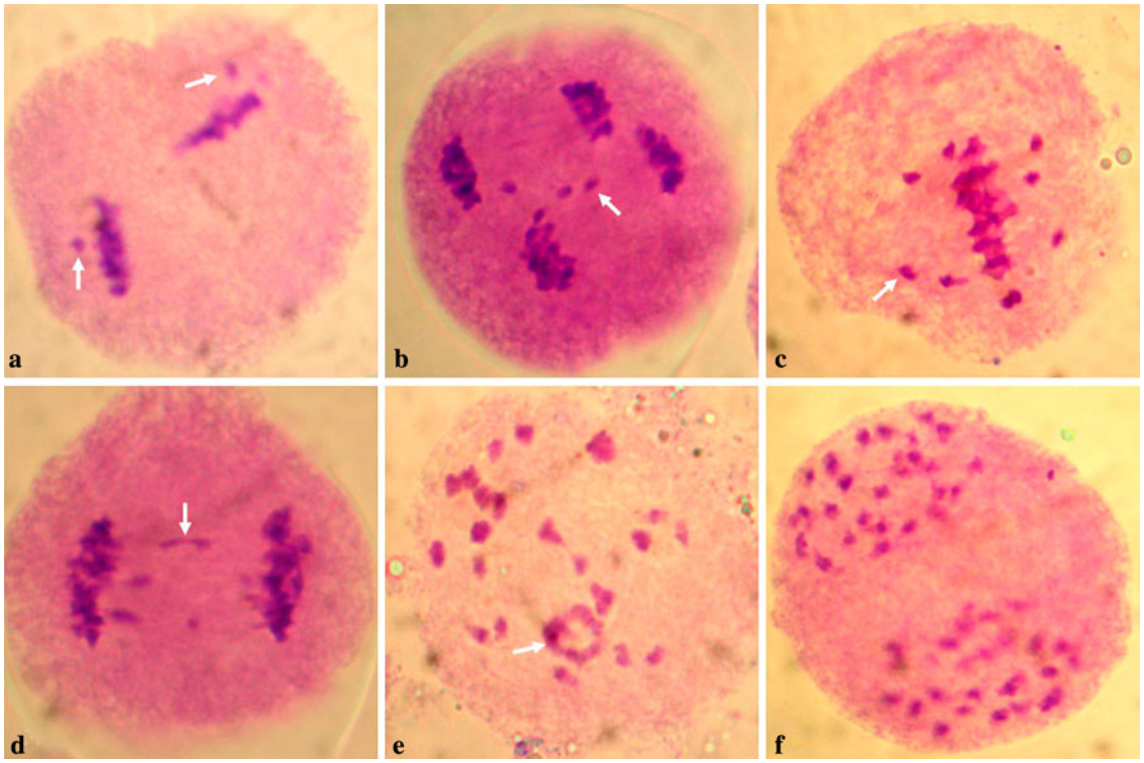

Fig. 5 Meiotic abnormalities. **a**, **b** Precocious chromosome migration at M II and laggards at A II in H_2 generation, precocious migration to both poles at M I (**c**), a chromosome bridge at A I (**d**), a quadrivalent at diakinesis (**e**), normal A I in H_3 generation (**f**)

Table 5 Genome stability shown with abnormal sequence changes in the three early generations of hexaploids evaluated with molecular markers

	Total observed alleles	Alleles lost		Novel alleles		Index of genome stability[b]
		Number	%[a]	Number	%	
Triploid	1,767	–	–	–	–	–
H_2	1,755	56	3.17	44	2.51	17.61a[d]
H_3	1,722	41	2.34	52	3.02	18.66a
H_4	1,718	31	1.80	27	1.57	29.67b
A genome	1,511[c]	44	2.91	47	3.11	16.61a[d]
C genome	461	13	2.82	12	2.60	18.44a
B genome	286	2	0.70	2	0.70	71.46b

[a] The ratio between the number of disappeared alleles in each generation and the number of observed alleles in its parental generation

[b] 1/(the percentage of disappeared alleles + the percentage of novel appearing alleles) × 100

[c] The total observed alleles in H_2, H_3 and H_4 generations

[d] Means of index of genome stability followed by the same letter are not significantly different according to Duncan's multiple range test ($p = 0.05$)

(Comai et al. 2000; Liu et al. 2001; Zhang et al. 2004). Nevertheless, many trait variations and genome changes such as low sterility, low embryonic viability (Madlung et al. 2005), phenotypic instability (Thompson and Lumaret 1992; Husband and Schemske 2000; Pires et al. 2004), DNA sequence changes (Song et al. 1995; Liu et al. 1998a, b; Comai 2000), epigenetic changes (Matzke et al. 1999; Levy and Feldman 2004; Wang et al. 2004), transposon activation (Singer et al. 2001; Kashkush et al. 2002) and even chromosome loss (Burnham 1962; Doyle 1986) can occur. However, for artificial de novo polyploids, which do not occur in nature, chromosomal instability is a major problem. The hexaploid Triticale (AABBRR) is a successful artificial allopolyploid crop that was obtained by

crossing tetraploid wheat (AABB) with rye (RR) (Ammar et al. 2004). The instability of this hybrid has caused serious reproductive disorders, such as a high frequency of aneuploids and low fertility, in initial generations (Nakajima 1954; Merker 1973a; Maich and Ordóñez 2003). The chromosomal stability and fertility of neopolyploids were found to be associated with genetic differences between parental lines (Tsuchiya and Larter 1971; Merker 1973b), and these factors were improved greatly in later generations when lines from different genetic backgrounds were intercrossed and selected (Larter and Gustafson 1980).

Even with stable chromosome numbers, the newly synthesised polyploids might be unstable due to drastic changes in their genomic DNA sequence. According to a range of analyses on different plants with molecular markers, the ratio of parental DNA fragments loss in resynthesised polyploid varied greatly, from 14.0% in wheat (Shaked et al. 2001) and 11.3% in *Spartina* (Salmon et al. 2005) to a relatively lower level in *B. napus* (1.8%; Lukens et al. 2006) and *Arabidopsis* (0.1%; Madlung et al. 2005). However, the ratio of parental DNA sequence loss in H_4 generations of de novo *Brassica* hexaploids in this experiment was only 1.80%, as low as in resynthesised *B. napus*. Genome changes may be caused by multivalent formation and intergenomic recombination (Ozkan 2000; Shaked et al. 2001). We found the frequency of multivalents was positively correlated with the ratio of sequence elimination across early generations of hexaploids. However, other unknown mechanisms might be involved in promoting the neopolyploids to a stable status (Song et al. 1995; Feldman et al. 1997).

Although several types of *Brassica* hexaploids (including that between *B. carinata* and *B. rapa*) have been obtained for the purpose of transferring genes or traits from alien species to the tetraploid *B. napus* (Sacristán et al. 1989; Gerdemann et al. 1994; Arumugam et al. 1996; Meng et al. 1998; Sjödin and Glimelius 1998; Li et al. 2004; Prakash et al. 2009), there is little information available about hexaploid progeny after the H_1 generation, with instability perhaps being the main reason. The three basic genomes in *Brassica* may coexist harmoniously with each other in the three tetraploid species; however, the situation is very different in de novo hexaploid *Brassica*. The frequency of allelic loss/gain detected with SSR markers was four times greater in the A and C genomes than in the B genome. Much evidence had been provided that genomes A/C exhibit closer homology as compared to A/B or C/B in *Brassica* (Attia and Röbbelen 1986; Truco et al. 1996; Chèvre et al. 1997), which could promote frequent homeologous exchanges between A and C genomes (Sharpe et al. 1995; Osborn et al. 2003; Udall et al. 2005) that lead to their instability. A/C homeologous pairing is proposed to suppress by the *PrBn* genes as reported for *B. napus* (Jenczewski et al. 2003; Liu et al. 2006), while in *Brassica* hexaploids the *PrBn* suppressing system could not be well established by the events arising from the genomic shock of hexaploidisation. For example, transposon activation per se may cause various types of genomic instability. Intercrossing different hexaploid lines with high genome stabilities would pyramid *PrBn* genes, and maintaining selection for the hexaploid plants with the highest genome stability in following generations would lead to a stable hexaploid *Brassica* species.

In the present study, large-scale interspecific hybridisation and selection for chromosome stability were carried out with the goal of establishing a hexaploid *Brassica* species (AABBCC). Although the frequency of hexaploid plants in the H_2 generation was extremely low (4.6%), it increased substantially to 23.0% in H_3 and 26.3% in H_4 following intensive selection. It is expected that this trend will continue, and the frequency of hexaploids will increase to a high level in the successive generations. Hexaploid lines with higher levels of chromosomal stability, such as C15 (with 56% hexaploids in H_4) and C21 (with the highest number of hexaploid plants in H_2 among all combinations; see Table 2), may then be crossed with each other to generate progeny with enhanced chromosomal stability. Moreover, the index of genome stability, first introduced in this study, significantly increased in early hexaploid generations and especially in H_4, showing a significant improvement in genome stability of *Brassica* hexaploids with better parental genotypic combinations. However, one could look to other sources of hexaploids to seek stable higher polyploid species of *Brassica*, e.g., *B. napus* × *B. nigra* as has recently been reported (Pradhan et al. 2010).

Once a stable AABBCC *Brassica* hexaploid is established, it should provide opportunities to analyse interactions between the three basic genomes of the genus *Brassica* and to investigate *Brassica* evolution. In addition, the mechanisms of chromosomal stability in *Brassica* hexaploids can be studied in detail through the construction of a mapping population using parents with different chromosomal stabilities, as was done for *B. napus* (Jenczewski et al. 2003; Liu et al. 2006). Moreover, valuable traits such as drought and disease resistance from two cultivated species, *B. carinata* and *B. rapa*, are combined in the hexaploid itself, and new transgressive traits might be created and selected by breeding. The particular origins and genome composition of the *Brassica* hexaploid enable it to be used as a bridge to transfer genes or traits to *Brassica* crops such as *B. napus*, *B. juncea* and *B. carinata*; this transfer is usually blocked by interspecific reproductive barriers.

Polyploid species themselves possess advantages over their lower ploidy parental species. The genomes of polyploid plants, with their fixed heterosis, usually have higher

plasticity and genetic diversity than those of the parents (Mahy et al. 2000; Soltis et al. 2004; Leitch and Leitch 2008). The *B. carinata* × *B. rapa* derived hexaploid could be used as a first stable hexaploid crop of *Brassica* in areas where the diploid and tetraploid crops do not perform well.

When the traditional natural *B. napus* was crossed with a new semisynthetic type, in which the A and C genomes were introduced from *B. rapa* and *B. carinata*, respectively, strong intersubgenomic heterosis was observed (Meng et al. 1998; Qian et al. 2005). The heterosis would be stronger if an additional pair of subgenomes was involved. Intersubgenomic hybrids could be produced at higher levels of ploidy by crossing *B. carinata* × *B. rapa* derived hexaploid to two other types of *Brassica* hexaploid (i.e., *B. napus* × *B. nigra* and *B. juncea* × *B. oleracea*), and stronger intersubgenomic heterosis is anticipated to occur between each pair of *Brassica* hexaploid types than is observed between tetraploids.

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