Cytoplasmic and Genomic Effects on Meiotic Pairing in *Brassica* Hybrids and Allotetraploids from Pair Crosses of Three Cultivated Diploids

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ABSTRACT Interspecific hybridization and allopolyploidization contribute to the origin of many important crops. Synthetic *Brassica* is a widely used model for the study of genetic recombination and "fixed heterosis" in allopolyploids. To investigate the effects of the cytoplasm and genome combinations on meiotic recombination, we produced digenomic diploid and triploid hybrids and trigenomic triploid hybrids from the reciprocal crosses of three *Brassica* diploids (*B. rapa*, AA; *B. nigra*, BB; *B. oleracea*, CC). The chromosomes in the resultant hybrids were doubled to obtain three allotetraploids (*B. juncea*, AA.BB; *B. napus*, AA.CC; *B. carinata*, BB.CC). Intra- and intergenomic chromosome pairings in these hybrids were quantified using genomic *in situ* hybridization and BAC-FISH. The level of intra- and intergenomic pairings varied significantly, depending on the genome combinations and the cytoplasmic background and/or their interaction. The extent of intragenomic pairing was less than that of intergenomic pairing within each genome. The extent of pairing variations within the B genome was less than that within the A and C genomes, each of which had a similar extent of pairing. Synthetic allotetraploids exhibited nondiploidized meiotic behavior, and their chromosomal instabilities were correlated with the relationship of the genomes and cytoplasmic background. Our results highlight the specific roles of the cytoplasm and genome to the chromosomal behaviors of hybrids and allopolyploids.

POLYPLOIDY has played a crucial role in the evolutionary history of higher plants. Up to 80% of flowering plant species have been estimated to have undergone one or more polyploidization events in their ancestry (Masterson 1994; Ramsey and Schemske 1998; Otto 2007; Wood *et al.* 2009). Interspecific hybridization and allopolyploidization contribute to the origin of many important crops, including canola (*Brassica*), cotton (*Gossypium*), tobacco (*Nicotiana*), and wheat (*Triticum*). Among the six *Brassica* crops in the U-triangle (Nagaharu 1935), *Brassica carinata* Braun ($2n = 34$, BBCC), *B. juncea* (L.) Czern. ($2n = 36$, AABB), and *B. napus* L. ($2n = 38$, AACC) are allotetraploids, which originated naturally through convergent alloploid evolution between any two of the three diploid species *B. nigra* (L.) Koch ($2n = 16$, BB), *B. oleracea* L. ($2n = 18$, CC), and *B. rapa* L. (syn. *B. campestris*, $2n = 20$, AA). This complex of diploids and allopolyploids is now considered as a model system for studying polyploidization in crop species (Lukens *et al.* 2006; Pires *et al.* 2006). Synthetic *Brassica*, especially *B. napus*, has become one of the most widely used models to study the genetic and epigenetic alterations caused by meiosis-driven genome reshuffling in allopolyploids (Gaeta *et al.* 2007; Nicolas *et al.* 2007, 2009; Szadkowski *et al.* 2010, 2011; Xiong *et al.* 2011) since the seminal work of Song *et al.* (1995).

Previous studies suggested that the genomes from two ancestral diploids in natural *Brassica* allotetraploids have different stabilities and that cytoplasm has exerted considerable influence on the evolution of nuclear genomes of alloploids (Prakash *et al.* 2009). It has been confirmed that *B. nigra* and *B. rapa* have contributed the cytoplasm to *B. carinata* and *B. juncea*, respectively. However, it is still uncertain about the cytoplasm donor of *B. napus*. *B. rapa* has been suggested as a potential plastid genome donor to *B. napus*

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doi: 10.1534/genetics.112.140780
Manuscript received February 20, 2012; accepted for publication April 7, 2012
Supporting information is available online at http://www.genetics.org/content/suppl/2012/04/13/genetics.112.140780.DC1.
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(Flannery *et al.* 2006; Allender and King 2010). When the parental diploid species of allopolyploid has highly differentiated cytoplasm, as in *B*. *juncea* and *B*. *carinata*, the nuclear genomes contributed by the male parents are considerably altered compared to the nuclear genomes of female parents (Song *et al.* 1988, 1995). The A genome in *B*. *juncea* has remained mostly intact while the B genome has changed considerably; the B genome in *B*. *carinata* has unchanged but the C genome has considerably altered. In *B*. *napus*, both A and C genomes have undergone a similar extent of changes. Nevertheless, recent comparative sequence analysis between homeologous genome segments of *B*. *napus* and its two progenitor species showed that the C-genome segments were expanded in size relative to their A-genome counterparts in the majority of the genomic regions studied and revealed that the C genome is more vulnerable to undergoing changes than the A genome after the formation of *B*. *napus* (Cheung *et al.* 2009). The cytoplasm background of resynthesized *B*. *napus* from its progenitors significantly affects the transmission frequency of the meiotic-driven genetic changes to the progenies (Szadkowski *et al.* 2010).

In spite of sharing considerable homeology between the partaking genomes, these *Brassica* allopolyploid species exclusively exhibit diploid-like meiosis. It has been proposed that diploid-like meiosis is genetically regulated in *Brassica* and its related genera (Prakash *et al.* 2009). However, so far only the major gene *PrBn* (**P**airing **r**egulator in ***B**rassica* ***n**apus*) in *B*. *napus* was demonstrated to be responsible for inhibiting the homeologous pairing in its haploids (Jenczewski *et al.* 2003), which has been mapped on linkage group C9, and to display incomplete penetrance (Liu *et al.* 2006). It was further shown that the variation in crossover frequency among *B*. *napus* accessions representing a range of genetic and geographic origins roughly correlates with the multiple origins of *B*. *napus* and *PrBn* diversity (Cifuentes *et al.* 2010). The findings highlight the diverse nature of homeologous recombination regulation in the wild. The diploid-like meiotic behavior of allopolyploids is also thought to result from the divergence between homeologous chromosomes, which may already exist and/or be accentuated at the onset of polyploid formation (Le Comber *et al.* 2010), and involve the rearrangement of large chromosome fragments (reviewed in Jenczewski and Alix 2004).

Genome structure and genomic relationships of the six *Brassica* species have been extensively investigated in the diversely originated natural types and artificially synthesized *Brassica* crop species (for review see Prakash *et al.* 2009), which, however, makes it difficult to compare the results from different reports because the extent of homeologous recombination in resynthesized *B*. *napus* is influenced by progenitor genotype and/or combination (Prakash and Hinata 1980; Attia *et al.* 1986) and cytoplasm backgrounds (Szadkowski *et al.* 2010). In the present investigation, the di- and trigenomic hybrids were produced from the reciprocal pair crosses of three *Brassica* diploids to study and compare the precise effects of genome combinations and cytoplasm on the meiotic recombination of each genome through genomic *in situ* hybridization (GISH) and BAC-FISH. The chromosome pairing in the synthetic *Brassica* allotetraploids showed nondiploidized behavior and was related to the genome affinity and cytoplasmic background. Our results provide new insight into the effects of the cytoplasm background and genome combinations on the chromosomal recombination in *Brassica* hybrids and allopolyploids and, more importantly, on the chromosome stability and diploidization in synthetic allopolyploids.

Materials and Methods

Plant material and crosses

Each genotype or cultivar of three cultivated *Brassica* diploids was used as parents in reciprocal crosses to produce interspecific hybrids: *B*. *rapa* ($2n = 20$, AA genome, genotype 3H120), *B*. *nigra* (L.) Koch cv. Giebra ($2n = 16$, BB), and *B*. *oleracea* var. *alboglabra* L. ($2n = 18$, CC, genotype Chi Jie Lan) (Figure 1). These materials were grown in the experimental fields on the campus of Huazhong Agricultural University, Wuhan, China. One plant in each genotype was selected for the reciprocal crosses performed by hand emasculation and pollination. After $\sim$20 days of pollination, the immature embryos were cultured on MS medium (Murashige and Skoog 1962). To ensure the homogenous identity of the hybrid plants from the same combination, the plantlet from one embryo of each cross was successively subcultured on MS medium with 1.5 mg/liter^{-1} 6-benzyl aminopurine (6-BA) and 0.25 mg/liter^{-1} α-naphthalenacetic acid (NAA) to generate enough cloned plantlets for study. The cloned plantlets were cultured on MS agar medium with 1.5 mg/liter^{-1} 6-BA, 0.25 mg/liter^{-1} NAA, and 100 mg/liter^{-1} colchicine for $\sim$10 days to double the number of chromosomes for synthesizing the allotetroploids and subsequently transferred to MS medium without colchicine until plantlets were regenerated from callus. Three allotetraploids (AA.BB, AA.CC, and BB.CC) were synthesized by doubling the chromosome numbers of the respective digenomic hybrids (see below), and CC.AA was directly obtained from the cultured embryo-plantlet, probably by the spontaneous chromosome doubling *in vitro*.

Cytological investigation and pollen fertility analysis

The ovaries from young flower buds were collected and treated with 8-hydroxyquinoline for 3–4 hr at room temperature before fixed in Carnoy's solution I (3:1 ethanol:glacial acetic acid, v/v) and stored at –20° for further chromosome counting in somatic cells. The young flower buds were fixed directly in Carnoy's solution I and stored at $-20°$ for meiosis study. Cytogenetic observation was carried out according to the methods of Li *et al.* (1995). More than 300 pollen grains from three flowers of each plant were stained with acetocarmine (1%), and the percentage of stainable pollen grains was calculated to measure pollen fertility.

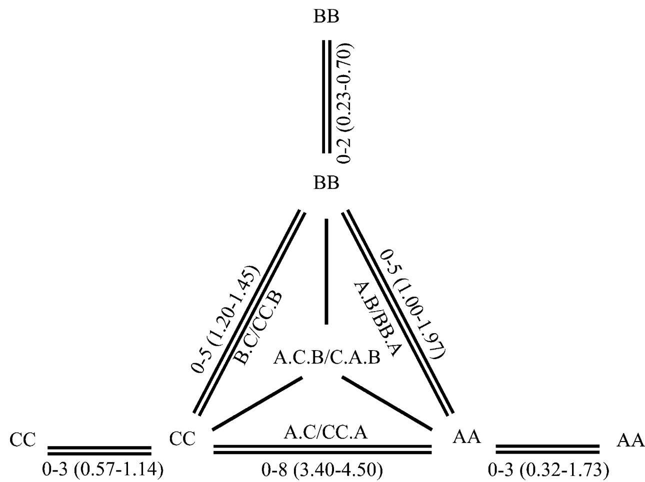

Figure 1 Schematic illustration showing the production of hybrids from pair crosses of three *Brassica* diploids and their chromosome pairing. AA, BB, and CC designate *B. rapa* (AA genome), *B. nigra* (BB genome), and *B. oleracea* (CC genome). The digenomic diploid or triploid hybrids obtained are indicated between two parents, and the trigenomic triploids are at the center of the triangle. The ranges and averages of allosyndetic bivalents between three pairs of genomes in digenomic and trigenomic hybrids are shown on the double lines connecting two parents, and those of autosyndetic bivalents are shown on the double lines connecting the same genome at the three joints.

Probes and chromosome preparations

The probes used for GISH and BAC-FISH were the following: (1) total genomic DNA of *B. nigra* cv. Giebra labeled with biotin-11-dUTP (Fermentas) by nick translation; (2) the plasmid DNA of BAC BoB014O06 (provided by Susan J. Armstrong, University of Birmingham, Birmingham, UK) labeled with biotin-11-dCTP (for A.C and CC.A hybrids) by random priming using the Bio-Prime DNA Labeling System kit (Invitrogen, Life Technologies) or with digoxigenin-11-dUTP (Roche, Basel, Switzerland) (for A.C.B combination) by random priming using the BioPrime Array CGH Genomic Labeling System kit according to the manufacturer's protocol (Invitrogen, Life Technologies).

Total genomic DNA of *B. rapa* (3H120) and 45S rDNA was boiled for 15 min twice to obtain DNA fragments of 100–500 bp and used as blocks. The 45S rDNA was used to replace the intergenic spacer of the *B. oleracea* 45S rDNA as the block agent (Howell *et al.* 2008) to reduce the intensity of strong signals and to produce a more even distribution of hybridization signal intensity. The C_0t-1 DNA was prepared from *B. oleracea* A12DHd genomic DNA (Zwick *et al.* 1997). Chromosome preparation for FISH was carried out according to Zhong *et al.* (1996) with minor modifications (Ge and Li 2007); an enzyme mixture containing 0.6% cellulase Onozuka RS (Yakult, Tokyo), 0.2% pectinase (Merck, Darmstadt, Germany), and 0.5% snailase (Beijing Baitai Biochem, Beijing) was used for digestion.

GISH and BAC-FISH

Slides were pretreated with 0.01% pepsin in 10 mM HCl for 20 min and with RNase in 2× SSC (DNase-free, 100 μg/ml, 1 hr at 37°), fixed in 4% paraformaldehyde for 10 min, dehydrated in 75 and 100% ethanol for 3 min each, and air dried. Hybridization mixture contained 50% deionized formamide, 2× SSC, 10% dextran sulfate, 0.5% SDS, and 100-ng probes for each slide, while ~1000 ng blocking A-genome DNA, 1 μg C_0t-1 DNA to block repeated sequence, and 100 ng 45S rDNA were added for blocking in A.C, CC.A, and A.C.B hybrids. The mixture was denatured at 70° for 10 min. The probes and chromosomal DNA on the slides were co-denatured at 80° for 5 min in a thermal cycler and hybridized at 37° overnight in a humid chamber. Slides were washed stringently for 10 min in 0.1× SSC with 20% deionized formamide at 42°. The immunodetection of biotinylated and digoxigenated DNA probe was carried out by using Cy3-labeled streptavidin (KPL, St. Louis) and anti-digoxigenin conjugate-FITC (Roch, Basel, Switzerland), respectively. Finally, preparations were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) solution (Roche, Basel, Switzerland) (1 mg/ml), mounted in antifade solution (Vector Laboratories, Peterborough, UK).

Image capturing, processing, and statistical analysis

All images were captured with a CCD camera attached to a fluorescence microscope (Nikon Eclipse 80i). Images were processed by Adobe Photoshop (Adobe Systems, San Jose, CA) to adjust contrast and brightness. Two-by-two chi-square contingency tests were used to test the difference in pairing configuration.

Results

Morphology, cytology, and pollen fertility in hybrids

From reciprocal pair crosses of three *Brassica* diploids, the following digenomic diploid and triploid hybrids were produced: A.B/BB.A, A.C/CC.A, and B.C/CC.B (Figure 1). The production of hybrids (BB.A, CC.A, and CC.B) likely resulted from the fusion of unreduced gametes by the female parent and reduced gametes by the male parent in the three crosses, although the possibility of chromosome duplication for one genome during the mitotic divisions of the zygote after fertilization could not be excluded. Two trigenomic hybrids (A.C.B and C.A.B) were obtained from the crosses between the synthesized *B. napus* (AA.CC/CC.AA) and *B. nigra*, which contained the cytoplasm of *B. rapa* and *B. oleracea*, respectively. No hybrids were obtained from the crosses synthetic *B. juncea* (AA.BB) × *B. oleracea* and synthetic *B. carinata* (BB.CC) × *B. rapa*, although many pollinations were carried out. The crossability between the synthesized allotetraploids and the diploids was quite low.

These hybrids generally showed an intermediate morphology, while the reciprocal hybrids inclined more to the maternal parents, especially BB.A, CC.A, and CC.B, probably due to the one more copy of the maternal genome (Supporting Information, Figure S1). The three digenomic hybrids (A.B, A.C, B.C) had very low pollen fertility, and the

two trigenomic hybrids (A.C.B, C.A.B) were male sterile. Among the three digenomic triploids (BB.A, CC.A, CC.B), BB.A and CC.B had much higher pollen fertility than CC.A and produced some seeds after open pollination. The pairing frequency in A.C was obviously higher than in A.B and B.C (Table S1), indicating the close homology between A and C genomes. The average of bivalents in C.A.B was significantly lower than that in A.C.B ($\chi^2 = 927.48$, $P < 0.01$), indicating the cytoplasmic effect on the chromosome pairing. The pairing frequency in BB.A and CC.B was similar, but much lower than that in CC.A. The pairing differences in these hybrids were further analyzed using FISH to examine the intra- and intergenomic pairing.

Variable chromosome pairing in different genome combinations

Parental chromosomes in the A.B, B.C, BB.A, and CC.B hybrids were easily distinguished by using the labeled *B. nigra* genomic DNA probe and those in the A.C and CC.A hybrids by using the *B. oleracea* BoB014O06 probe. The chromosomes from A, B, and C genomes in the A.C.B hybrid were identified by dual-color FISH with the labeled *B. nigra* DNA and BoB014O06 probes. The BoB014O06 hybridized strongly to the centromeric region; therefore, 45S rDNA and C_0t-1 DNA blocking in hybridization mixture could reduce the intensity of the strong signals and produce an even hybridization signal intensity distribution. Additionally, the chromosome pairings within each genome (autosyndesis) and between different genomes (allosyndesis) were quantified and compared (Table 1 and Table 2).

In A.B, the average of univalents for A genome (4.74) was higher than that for B genome (3.53); the difference might result from the presence of two more chromosomes in the A genome than in the B genome because their rates of the univalents to the chromosome number of the genome were nearly the same. The maximum autosyndetic bivalent of the A genome was 3, one more than that of the B genome, and the average of the A genome (0.53) was higher than that of the B genome (0.23). The average and maximum of allosyndetic bivalents were 1.38 and 5, respectively—much higher than those of autosyndesis (Table 1). The trivalents A-A-B and A-B-B occurred in 37.7 and 26.23% of pollen mother cells (PMCs), respectively (Table S2). The total average chromosomes per cell and the rates for autosyndesis within the A or B genome were significantly lower than those for allosyndesis (Table 2). The average of the autosyndetic chromosomes in A genome (1.93) was higher than that in B genome (1.15), but the rates were similar. The same 3.33 chromosomes showed allosyndesis in the two genomes at similar rates (0.33 and 0.42). In A.C, the average and the rate of univalents for A (2.64/0.26) and C genomes (2.23/0.25) were comparable; the autosyndetic bivalents within A and C genomes were 1.18 and 1.14, with a maximum of 3.0. The average (3.45) and maximum (7.0) of allosyndetic bivalents were much higher than those of autosyndesis (Table 1). The trivalents A-A-C and A-C-C were

Table 1 Chromosome associations in PMCs of hybrids at diakinesis and metaphase I as revealed by FISH

		Chromosome associations (ranges)														
		I				II							III	IV		
Hybrids	2*n*	Total	I^{A}	I^{B}	I^{C}	Total	II^{A-A}	II^{B-B}	II^{C-C}	II^{A-B}	II^{A-C}	II^{B-C}	Total	Total	PMCs with multivalent (%)	Total PMCs
A.B	18	8.26 (3–16)	4.74^{a} (2–9)	3.53^{b} (1–7)	—	2.13 (0–5)	0.53^{b} (0–3)	0.23^{c} (0–2)	—	1.38^{c} (0–5)	—	—	0.75 (0–3)	0.70 (0–2)	86.89	61
A.C	19	4.86 (1–13)	2.64^{b} (0–8)	—	2.23^{a} (0–5)	5.77 (3–9)	1.18^{a} (0–3)	—	1.14^{b} (0–3)	—	3.45^{c} (0–7)	—	0.86 (0–3)	—	83.05	22
B.C	17	9.43 (3–13)	—	4.72^{a} (1–7)	4.72^{d} (1–9)	2.10 (0–5)	—	0.30^{b,c} (0–2)	0.57^{c} (0–2)	—	—	1.23^{c} (0–5)	0.65 (0–3)	0.30 (0–1)	74.17	120
A.C.B	27	5.27 (2–8)	0.64^{c} (0–2)	4.32^{a} (2–7)	0.32^{c} (0–2)	10.32 (2–12)	1.73^{d} (0–3)	0.45^{b,a} (0–1)	1.14^{b} (0–3)	1.00^{c} (0–3)	4.50^{b} (1–8)	1.50^{c} (0–5)	0.41 (0–1)	—	40.91	22
BB.A	26	7.21 (3–10)	5.65^{d} (1–9)	1.56^{c} (0–6)	—	8.12 (6–10)	0.65^{b} (0–2)	5.35^{d} (2–7)	—	2.12^{b} (0–5)	—	—	0.85 (0–2)	—	67.65	34
CC.A	28	5.91 (3–10)	5.18^{a,d} (2–9)	—	0.74^{c} (0–4)	10.38 (8–12)	0.32^{c} (0–2)	—	6.29^{a} (2–9)	—	3.76^{c} (0–8)	—	0.44 (0–1)	—	44.12	34
CC.B	26	6.40 (4–14)	—	4.80^{a} (3–7)	1.60^{b} (0–7)	8.70 (6–10)	—	0.70^{a} (0–1)	6.70^{a} (5–8)	—	—	1.20^{c} (0–3)	0.80 (0–2)	—	70	10

I, univalent; II, bivalent; III, trivalent; IV, quadrivalent. I^{A}, I^{B}, and I^{C} indicate univalents belonging to the A, B, and C genomes, respectively; II^{A-A}, II^{B-B}, and II^{C-C} indicate autosyndetic bivalents formed between two chromosomes of A, B, or C genomes in A.B, A.C, B.C, and A.C.B hybrids, but II^{B-B} in BB.A and II^{C-C} in CC.A and CC.B indicate homologous bivalents; II^{A-B}, II^{A-C}, and II^{B-C} indicate allosyndetic bivalents formed between A and B chromosomes, A and C chromosomes, and B and C chromosomes, respectively.
^{a,b,c,d}Groups significantly different by χ^2 -test, $p < 0.05$.

Table 2 Means of chromosome numbers and ratios for no pairing, autosyndesis, and allosyndesis within each genome in hybrids

	A genome			B genome			C genome		
Hybrids	No pairing	Autosyndesis	Allosyndesis	No pairing	Autosyndesis	Allosyndesis	No pairing	Autosyndesis	Allosyndesis
A.B	4.74 (0.47^{a})	1.93^{b} (0.19^{b,*})	3.33^{b} (0.33^{c,**})	3.53 (0.44^{b})	1.15^{c} (0.14^{c,*})	3.33^{b} (0.42^{b**})	—	—	—
A.C	2.64 (0.26^{b})	3.50^{a} (0.35^{a,*})	3.86^{b} (0.39^{c,**})	—	—	—	2.23 (0.25^{b})	2.91^{d} (0.32^{b,*})	3.86^{b} (0.43^{b,**})
B.C	—	—	—	4.72 (0.59^{a})	1.23^{c} (0.15^{c,*})	2.06^{c} (0.26^{c,**})	4.72 (0.53^{a})	2.23^{a} (0.25^{b,c,*})	2.06^{c} (0.23^{c,**})
A.C.B	0.64 (0.06^{c})	3.68^{a} (0.37^{a,*})	5.68^{a} (0.57^{b,**})	4.32 (0.54^{a,b})	1.00^{c} (0.13^{c})	2.68$^{c,\ b}$ (0.34^{c,b})	0.32 (0.04^{c})	2.36^{a,d} (0.26^{b,c,*})	6.32^{a} (0.70^{a,**})
BB.A	5.65 (0.57^{a})	1.68^{b} (0.17^{b,c,*})	2.68^{c} (0.27^{c,**})	1.56 (0.20^{c})	1.06^{c} (0.13^{c,*})	2.68^{b,c} (0.34^{b,c,**})	—	—	—
CC.A	5.18 (0.52^{a})	0.74^{c} (0.07^{c,*})	4.09^{b} (0.41^{c,**})	—	—	—	0.74 (0.08^{c})	0.59^{c} (0.07^{c,*})	4.09^{b} (0.45^{b,**})
CC.B	—	—	—	4.80 (0.60^{a})	1.40^{c} (0.18^{c,*})	1.80^{c} (0.23^{c,**})	1.60 (0.18^{b,c})	1.20^{b} (0.13^{b,c,*})	1.80^{c} (0.20^{c,**})
Average	4.21 (0.42)	2.07 (0.21*)	3.72 (0.37**)	4.34 (0.54)	1.19 (0.15*)	2.47 (0.31**)	3.79 (0.42)	2.34 (0.26*)	2.87 (0.32**)

For the calculation of the total chromosome numbers involved in autosyndesis within each genome in hybrids (A.B, A.C, B.C, A.C.B), the bivalent, trivalent, and quadrivalent of the same genome mean two, three, and four chromosomes, while the trivalent of two genomes, such as A-A-C, is taken as 1.5 A-genome chromosomes for autosyndesis and 0.5 A-genome chromosome and 1 C-genome chromosome for allosyndesis, and the quadrivalent (A-A-A-C) is divided into 2.5 A-genome chromosomes for autosyndesis and 0.5 A-genome chromosome and 1 C-genome chromosome for allosyndesis. For the calculation of the total chromosome number for allosyndesis within each genome, the bivalent means one chromosome for one of two genomes, the chromosome shared by two associations in trivalent or quadrivalent is taken as one-half for one genome and another half for the other genome. The ratios of autosyndesis, allosyndesis, and no pairing within each genome are the observed chromosome numbers/the total chromosome numbers of each genome. In digenomic triploid hybrids (BB.A, CC.A, and CC.B), the homologous bivalents in Table 1 are excluded from the chromosomes for autosyndesis. For the division of pairing in trivalents, the B-B pairing in A-B-B of BB.A is assumed homologous pairing, and A-B pairing is allosyndetic pairing; for B-B-B, both homologous and autosyndetic pairings are assumed. The same calculation is made for CC.A and CC.B. Single (*) and double (**) asterisks indicate that the averages of autosyndesis or allosyndesis, respectively, from different genomes in each hybrid are insignificantly different (the chi-square test: $\alpha = 0.05$).
^{a,b,c}Groups significantly different by χ^2 -test, $p < 0.05$.

observed in 27.27 and 22.73% of PMCs, respectively (Table S2). Total numbers of chromosomes and rates for autosyndesis were 3.50 and 0.35 in the A genome and 2.91 and 0.32 in the C genome, which are similar numbers to those for allosyndesis, the same 3.86 chromosomes at the similar rates (0.39 and 0.43) (Table 2). In B.C, the univalent frequency for both B and C genomes was 4.72; the autosyndetic bivalents for B genome were 0.30, lower than 0.57 for C genome. The average and maximum of allosyndetic bivalents were 1.23 and 5.0, respectively—much higher than those of autosyndesis (Table 1, Figure 2C). The trivalents B-B-C and B-C-C appeared in 23.23% of PMCs (Table S2). The chromosomes for allosyndesis in B genome (2.06) were significantly higher than those for autosyndesis (1.23), but not for the rates (0.26, 0.15). The C genome had similar chromosomes for auto- and allosyndesis. The B and C genomes had different chromosomes (1.23 and 2.23) for autosyndesis, respectively, but the rates were similar (0.15 *vs.* 0.25). They happened to have the same 2.06 chromosomes for allosyndesis (Table 2). In A.C.B, the univalent frequencies for A and C genomes (0.64, 0.32) were much lower than for the B genome (4.32). Inversely, the autosyndetic bivalents within A and C genomes (1.73, 1.14) were much higher than within the B genome (0.45). The average and maximum of allosyndetic bivalents were 4.50 and 8.0 for A.C, >1.00 and 3.0 for A.B, and 1.50 and 5.0 for B.C, respectively, (Table 1). Trivalents A-B-C and A-A-A were observed in 18.18 and 13.64% of PMCs (Table S2). As a whole, the chromosome frequencies for autosyndesis were significantly lower than those for allosyndesis in all three genomes. The chromosomes and rates for autosyndesis in A and C genomes (3.68/0.37 and 2.36/0.26) were similar but higher than those in B genome (1.00/0.13), and the values for allosyndesis in A and C genomes (5.68/0.57 and 6.32/0.70) were also similar but higher than those in B genome (2.68/0.34) (Table 2).

Among the three digenomic diploid hybrids A.B, A.C, and B.C, the average of bivalents in A.C was higher than that in A.B and B.C. Such difference resulted mainly from higher allosyndetic bivalents for A-C (3.45) than for A-B (1.38) and B-C (1.50) (Table 1, Figure 3B). Moreover, the bivalents and chromosomes of autosyndesis within A and C genomes in A.C was much higher than that for A genome in A.B and for C genome in B.C, respectively (Table 1 and Table 2). The average of bivalents in A.B and B.C was similar. In triploid A.C.B, the univalents were mainly from B genome (Tables 1 and 2; Figure 3A). The autosyndetic bivalents and chromosomes within A and C genomes were similar to those in A.C, but autosyndesis for B genome was similar to A.B and B.C (Table 1 and Table 2; Figure 3, B and C). The average of allosyndetic bivalents for A-B and B-C was comparable to A.B and B.C hybrids, respectively, and that for A-C was higher than in A.C. The total chromosomes involving in allosyndesis in A genome were significantly higher in A.C.B than in A.C and A.B, while those in A.C and A.B were similar. The values in C genome were also higher in A.C.B than in A.C

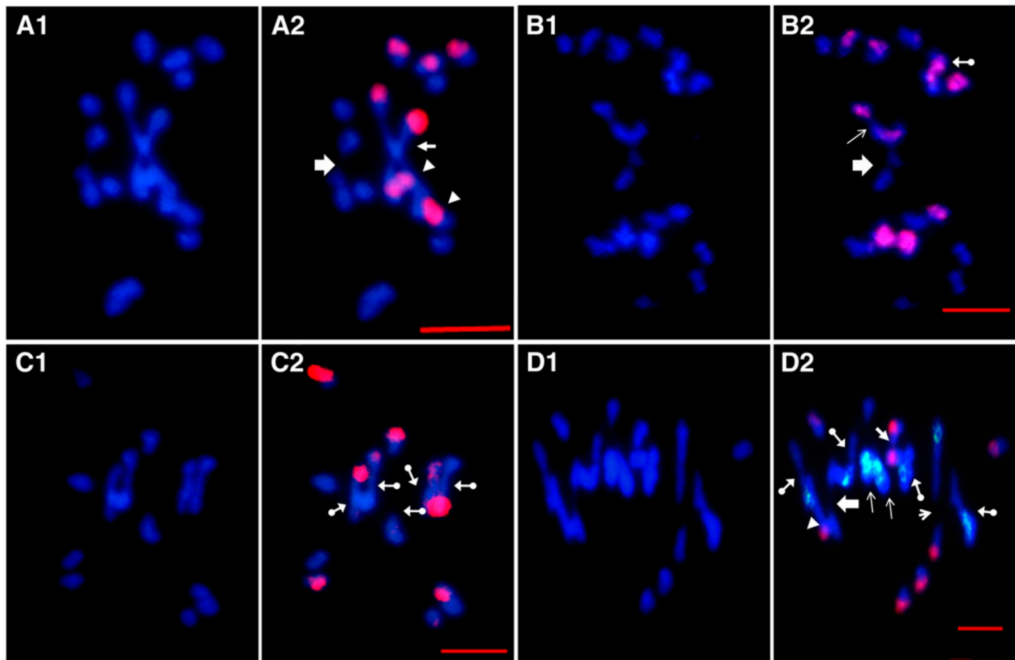

Figure 2 GISH/BAC-FISH analyses of meiotic chromosome pairings in PMCs of *Brassica* hybrids. DAPI (blue) and merged images are given for each cell. (A1 and A2) One diakinesis PMC from A.B with the following pairing: 6 I^{A} + 4 I^{B} + 1 $II^{A\text{-}A}$ (big arrow) + 1 $II^{B\text{-}B}$ (solid arrow) + 2 $II^{A\text{-}B}$ (solid arrowhead). Red signals are from labeled *B. nigra* probe. (B1 and B2) One diakinesis PMC from A.C with the following pairing: 7 I^{A} + 6 I^{C} +1 $II^{A\text{-}A}$ (open arrow) + 1 $II^{C\text{-}C}$ (arrow) + 1$II^{A\text{-}C}$ (solid arrows with ball tail). Red signals are from C genome specific BAC BoB014O06. (C1 and C2) One Metaphase I (MI) PMC from B.C with the following pairing: 3 I^{B} + 4 I^{C} + 5 $II^{B\text{-}C}$ (solid arrow with ball tail). Red signals are from *B. nigra* probe. (D1 and D2) One MI PMC from A.C.B with the following pairing: 3 I^{A} +4 I^{B} + 1 $II^{A\text{-}A}$ (big arrow) + 1 $II^{B\text{-}B}$ (solid arrow) + 2 $II^{C\text{-}C}$ (arrow) + 1 $II^{A\text{-}B}$ (solid arrowhead) + 4 $II^{A\text{-}C}$ (solid arrow with ball tail) + 1 $II^{B\text{-}C}$ (arrowhead). Red signals are from *B. nigra* probe, and green signals are from the C-genome-specific BAC BoB014O06. All bars, 5μm.

and B.C, and those in A.C were higher than in B.C. The values of B genome in A.C.B were similar to those in A.B or B.C, but the values in A.B were higher than in B.C (Table 2; Figure 3C). These pairing results not only showed that A and C genomes were more closely related than A and B genomes or B and C genomes, but also that the auto- and allosyndesis was suppressed or enhanced simultaneously. The pairing involving B genome was less affected, while that involving A or C genome was prone to more alterations.

In three digenomic triploid hybrids (BB.A, CC.A, CC.B), the chromosomes from one parent existed as homozygous pairs from chromosome duplication and others were in haploidy state. In BB.A, the average and maximum of autosyndetic bivalents within A genome were 0.65 and 2.0, respectively—fewer than those of A-B allosyndetic bivalents (2.12 and 5.0). The average and maximum of homologous bivalents within B genome were 5.35 and 7.0, which means that allosyndesis occurred in all cells. Trivalents A-B-B and B-B-B occurred in 38.24 and 17.65% of PMCs, respectively. The 1.68 chromosomes for autosyndesis in A genome were fewer than the 2.68 chromosomes for allosyndesis. Of 16 chromosomes of B genome, 10.70 showed homologous pairing, 1.06 autosyndetic pairing, 2.68 allosyndetic pairing, and 1.56 nonpairing (Table 2). In CC.A, the average and maximum of autosyndetic bivalents within A genome were 0.32 and 2.0, respectively, which is fewer than those of A-C allosyndetic bivalents (3.76 and 8.0). The average and maximum of homologous bivalents within C genome were 6.29 and 9.0, respectively. Trivalents A-C-C and C-C-C occurred in 26.47 and 11.76% of PMCs, respectively. The 0.74 chromosomes for autosyndesis in A genome were much fewer than 4.09 for allosyndesis. Among 18 chromosomes of C genome, 12.58 formed homologous bivalents, 0.60 and 4.09 gave auto- and allosyndesis, and 0.74 unpaired (Table 2). The autosyndetic bivalents within A genome in BB.A (0.65) was significantly higher than that in CC.A (0.32) and also for the chromosomes for autosyndesis (Table 2 and Table 3). However, the frequency of allosyndetic bivalents for A-B (2.12) was lower than that for A-C (3.76), and the chromosomes for allosyndesis in A genome in BB.A (2.68) were also fewer than 4.09 in CC.A (Table 2). In CC.B, the average and maximum of autosyndetic bivalents within B genome were 0.70 and 1.0, respectively, which were lower than those of B-C allosyndetic bivalents (1.20 and 3.0). The average and maximum of homologous pairing within C genome were 6.70 and 8.0, respectively. Trivalents B-C-C and C-C-C were observed in 50 and 10% of PMCs, respectively. The chromosomes for auto- and allosyndesis in B genome were similar (1.40 and 1.80, respectively). The associations of 18 chromosomes of C genome occurred as 13.40 for homologous pairing, 1.20 for autosyndesis, 1.80 for allosyndesis, and 1.60 for no pairing (Table 2). The number of chromosomes in C genome for allosyndesis (4.09) in CC.A were much higher than those in CC.B (1.80), while the chromosomes for autosyndesis in CC.A (0.59) were fewer than those in CC.B (1.20).

The autosyndetic bivalents and total chromosomes within A genome in A.B and BB.A had no significant difference, but the chromosomes of A genome for allosyndesis in BB.A were significantly lower than those in A.B (Table 2). This suggested that the haploid or diploid state of B genome affected mainly the allosyndetic pairing of A genome. The autosyndesis

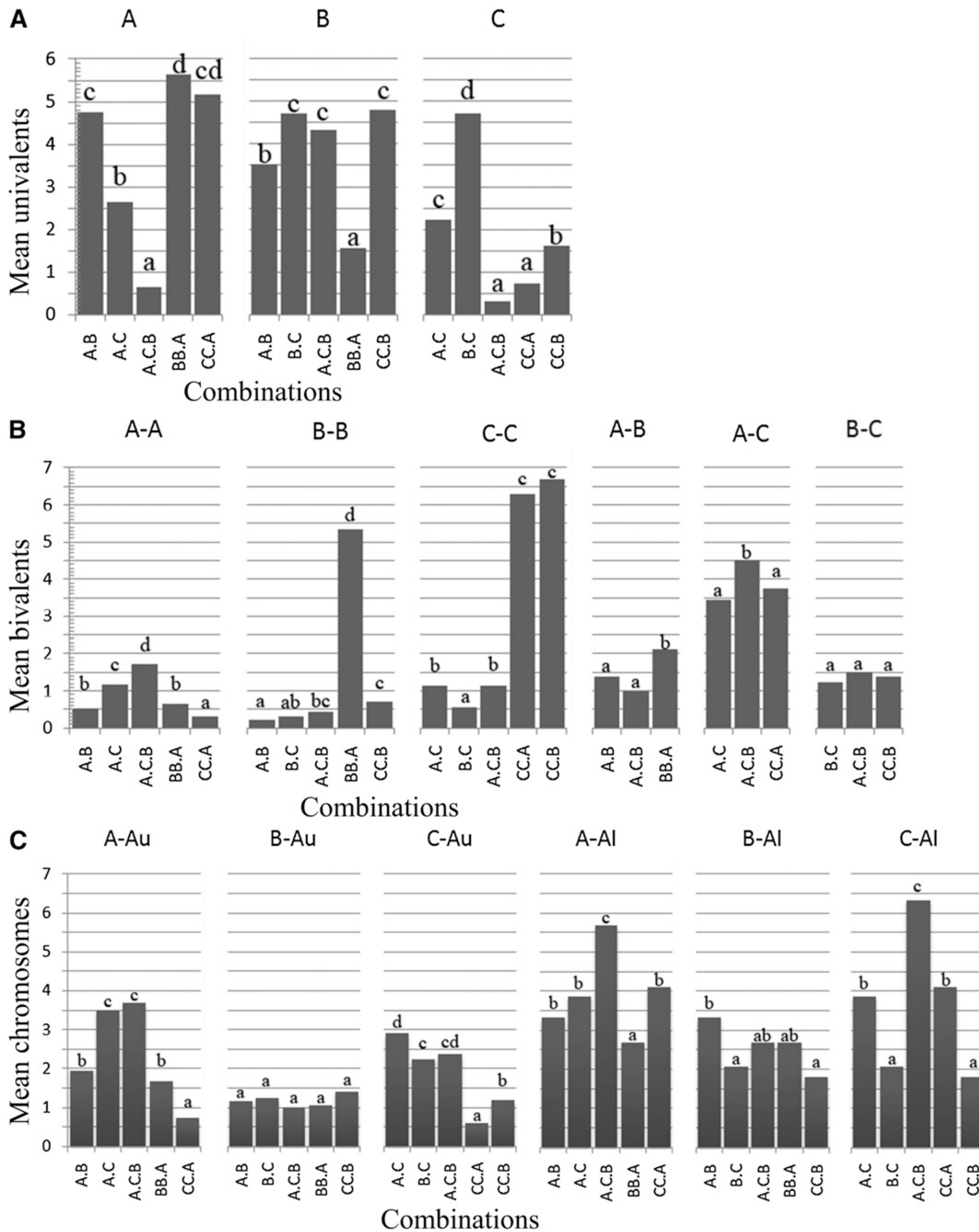

Figure 3 Comparisons of the averages of univalents within each genome (A), autosyndetic and allosyndetic bivalents in hybrids (B), and the averages of chromosomes involving autosyndesis and allosyndesis (Au, Al) within each genome across hybrids (C). Shared letters within each type denote that the values are not significantly different (contingent chi-square tests: α = 0.05).

within B genome and B-C allosyndesis in the two-hybrids B.C and CC.B were similar (Table 1 and Table 2), suggesting that the haploid or diploid state of C genome has no obvious effect on the pairing of B genome. The fewer C-genome univalents in CC.A than in CC.B suggested that the C-genome pairing was enhanced by A genome but reduced by B genome.

As A, B, and C genomes in these hybrids were from the same three diploids (Figure 1), the chromosome pairing of each genome could be compared, which should reveal the effects of different genome combinations and different types of cytoplasm on the chromosome pairing (Figure 3; Table 1, Table 2, and Table 3). The total chromosomes per cell involved in auto- and allosyndesis within B genome in these hybrids were generally lower than those in A and C genomes and varied in narrower ranges (Table 2, Figure 3C), but the rates of the total chromosome number of the three genomes were similar in most hybrids; only in A.C.B were the rates for auto- and allosyndesis within B genome significantly lower than those within A and C genomes (Table 2, Figure 3C). The averages and rates for autosyndesis in each hybrid were significantly lower than those for allosyndesis, but not for those of A and C genomes in A.C, of C genome in B.C, and of B genome in CC.B. The gross averages and rates for autosyndesis within each genome across all hybrids were also significantly lower than those of allosyndesis (Table 2). Although the average number of chromosomes for auto- and allosyndesis in B genome (1.19, 2.47) were fewer than

Table 3 Meiotic pairings and pollen fertility in synthetic allotetraploids

Allotetraploids	2*n*	I Means (ranges)	II Means (ranges)	III Means (ranges)	IV Means (ranges)	PMCs with multivalent (%)	Total PMCs	Pollen fertility (%)
AA.BB	36	0.04 (0–2)	17.85 (16–18)	—	0.08 (0–1)	7.09	141	88.20
AA.CC	38	0.45[a] (0–10)	18.64[a] (14–19)	—	0.07[a] (0–2)	4.49	89	83.89
CC.AA	38	0.36[a] (0–6)	18.64[a] (12–19)	0.01 (0–2)	0.10[a] (0–2)	11.68	182	33.33
BB.CC	34	0.73 (0–6)	16.62 (14–17)	—	0.01 (0–1)	0.73	137	60.52

I, univalent; II, bivalent; III, trivalent; IV, quadrivalent.
[a] Shared letters within each association type denote that the values are insignificantly different (the chi-square test: $\alpha = 0.05$)

those in A (2.07, 3.72) and C (2.33, 2.87) genomes, their rates were similar, *e.g.*, 0.21 and 0.37, 0.15 and 0.31, and 0.26 and 0.32 for A, B, and C genomes, respectively. Among the hybrids with haploid A genome (A.B, A.C, A.C.B, BB.A, and CC.A), the chromosomes and rates of autosyndesis within A genome in A.C.B and A.C were much higher than in BB.A and A.B and higher than in CC.A, except the rates between A.B and BB.A, and BB.A and CC.A. In A.B, A.C, and A.C.B with donor cytoplasm from *B. rapa*, those values in A. C.B and A.C with similar frequencies were significantly higher than that in A.B (Table 2). The difference was insignificant between A.B and BB.A, but significant between A. C and CC.A. This further showed that the haploid or diploid of B or C genome had different effects on the pairing of A genome. However, the chromosomes and rates for autosyndesis in B genome showed insignificant differences among the hybrids with haploid B genome (A.B, B.C, A.C.B, and CC.B), which were also similar to those in BB.A, after excluding homologous pairing (Table 2). This showed that the haploidy or diploidy of C genome had no or limited effects on the pairing of B genome. Among the hybrids with haploid C genome (A.C, B.C, A.C.B), the number of chromosomes for autosyndesis within C genome in A.C were higher than those in B.C, but not than those in A.C.B (Table 2), suggesting that the A and B genomes had different effects on influencing the homologous pairing of C genome. The autosyndetic chromosomes in C genome in these hybrids were much higher than those in CC.A and CC.B with dominant homologous pairing. The higher frequency in CC.B (1.20) than in CC.A (0.59) was possibly caused by the lower allosyndesis for the more distant relationship between B and C genomes than between A and C genomes.

For the allosyndesis within A genome, the chromosomes in A.C.B were higher than those in CC.A, A.C, and A.B and higher than in BB.A, and the rate in A.C.B was higher than that in the other four with no significant differences (Figure 1 and Figure 3C; Table 1). This result showed that the introduction of B genome could enhance the A-C association and also that a close homeology existed between A and C genomes because the haploid or diploid C genome did not change its homeologous pairing. For allosyndesis within B genome, the chromosomes in A.B were significantly higher than those in BB.A, B.C, and CC.B, but not in A.C.B; those in A.C.B and BB.A were higher than in B.C and CC.B, and the latter two had similar values. The rates in A.B, A.C.B, and BB.A were higher than those in B.C and CC.B. The A-B associations and the chromosomes for allosyndesis in A and B genomes in BB.A were significantly higher than in A.B, suggesting that the haploid or diploid B genome had obvious effect on the homeologous pairing of the two related genomes. The B-genome chromosomes for allosyndesis in B.C and CC.B were fewer than those in A.B. Once again, the similar frequencies in B.C and CC.B proposed that the haploid or diploid C genome had no obvious effect on the homeologous pairing of the two related genomes. For the allosyndesis within C genome, the chromosomes and rates in A.C.B were higher than those in CC.A and A.C and higher than B.C and CC.B, while those between CC.A and A.C or B.C and CC.B were similar. Notably, the allosyndesis frequency in A and C genomes was highest in A.C.B, while the frequency in B genome was somehow similar to those in other hybrids.

Chromosome pairing in synthetic allotetraploids

The chromosome pairings in the synthetic allotetraploids (AA.BB, AA.CC/CC.AA, and BB.CC) were not fully diploidized, and the univalents and multivalents appeared frequently (Table 3). The data from conventional and GISH observations (Table 3 and Table 4) showed that the average of univalents in AA.BB was significantly lower than in the other three, and that in AA.CC and CC.AA was lower than that in BB.CC, while the difference between AA.CC and CC. AA was insignificant ($\chi^2 = 0.95$, $P > 0.05$). The trivalents were observed only in CC.AA with low frequency. The frequencies of quadrivalents in AA.BB, AA.CC, and CC.AA were similar, but significantly higher than that in BB.CC. The differences among the bivalents were expected, considering their different chromosome numbers. The pollen fertility in these synthetics seemed to be negatively correlated with the frequency of univalents, while the different pollen fertilities between the reciprocal AA.CC and CC.AA (83.89/ 33.33%) were possibly attributable to the cytoplasmic effect, since their chromosome pairing behaviors were identical except for the different rates of PMCs with multivalents (4.49/11.68%) (Table 3).

With GISH/BAC-FISH analyses, the genome-specific homologous and homeologous pairings were distinguished (Table 4). The genome-specific univalents occurred in these allotetraploids except AA.BB. In both AA.CC and CC.AA, A-genome univalents were more frequent than those in C

Table 4 FISH studies of chromosome pairing in synthetic allotetraploids

		Chromosome associations (ranges)													
		I				II					III	IV			
Allotetraploids	2*n*	Total	I^A	I^B	I^C	Total	II^{A-A}	II^{B-B}	II^{C-C}	II^{A-C}	Total	Total	A-A-A-A	Others	Total PMCs
AA.BB	36	—	—	—	—	17.74 (16–18)	9.80^a (8–10)	7.94^a (7–8)	—	—	—	0.13 (0–1)	0.07^a (0–1)	A-A-B-B 0.06 (0–1)	69
AA.CC	38	0.52 (0–10)	0.35^a (0–6)	—	0.17^a (0–4)	18.22 (14–19)	9.52^b (6–10)	—	8.61^b (6–9)	0.09 (0–2)	—	0.26 (0–2)	—	A-A-C-C 0.26^b (0–2)	23
CC.AA	38	0.11 (0–8)	0.08^b (0–6)	—	0.03^b (0–2)	18.41 (12–19)	9.66$^{a,\ b}$ (5–10)	—	8.75^b (7–9)	—	0.03 (0–2)	0.25 (0–2)	0.03^b (0–1)	A-A-C-C 0.22^b (0–2)	73
BB.CC	34	0.67 (0–4)	—	0.46 (0–4)	0.21^a (0–2)	16.62 (15–17)	—	7.74^b (6–8)	8.87^b (8–9)	—	—	0.03 (0–1)	—	B-B-C-C 0.03 (0–1)	39

I, univalent; II, bivalent; III, trivalent; IV, quadrivalent. I^A, I^B, and I^C indicate univalents belonging to the A, B, and C genomes, respectively; II^{A-A}, II^{B-B}, and II^{C-C} indicate homologous bivalents formed between a pair of A or a pair of B or a pair of C chromosomes, respectively.

[a,b]Groups significantly different by χ^2 -test, $p < 0.05$.

genome. The mean of A-genome univalents in AA.CC was significantly higher than that in CC.AA. In BB.CC, B-genome univalents were more frequent than C-genome ones ($\chi^2 = 4.36$, $P < 0.05$). The C-genome univalents in CC.AA were significantly lower than in AA.CC and BB.CC, but the differences in AA.CC and BB.CC were insignificant. The averages of A-genome homologous bivalents in AA.CC and CC.AA were similar, but only the value in AA.CC was significantly lower than in AA.BB. The B-genome homologous bivalents in BB.CC were significantly lower than in AA.BB, which resulted from the occurrence of B-genome univalents in BB.CC, but not in AA.BB. The means of C-genome homologous bivalents were similar among AA.CC, CC.AA, and BB.CC. The homeologous bivalents and trivalents were observed only in AA.CC and CC.AA, respectively. The quadrivalents involving the chromosomes of one or two genomes were formed in these allotetraploids, their means varied (0.03–0.26), and the maximum was 1–2, being higher in AA.CC/CC.AA. The A-A-A-A quadrivalents occurred with the similar rates in AA.BB and CC.AA. The A-A-B-B quadrivalents appeared in AA.BB. The A-A-C-C quadrivalents occurred in AA.CC and CC.AA at relatively high rates (0.26 and 0.22), and two such pairings per cell were observed (Table 4, Figure 4B), but their difference was insignificant ($\chi^2 = 0.01$, $P > 0.05$). The B-B-C-C quadrivalents also occurred in BB.CC at a low rate. The lowest rates of C-genome-specific univalents within these synthetics and the similar rate of C-genome bivalents among these synthetics suggested that the C-genome chromosomes generally showed more normal pairing than those of A and B genomes (Table 4).

For the prevalence of homologous pairing in these allotetraploids, the averages of chromosomes involved in auto- and allosyndesis within each genome were low (0–0.35), but allosyndetic chromosomes in AA.CC and CC.AA were much higher (Table 5). In AA.BB, both pairings within A genome were observed with higher frequency for autosyndesis, but only allosyndesis within B genome at the same frequency as A genome was observed. In AA.CC, the two genomes showed only allosyndesis at the same frequency (0.35). In CC.AA, the two genomes showed auto- and allosyndesis, with the latter at much higher frequency. In BB.CC, only allosyndesis within two genomes occurred at low frequency (0.03). For the comparison of pairing within A genome, AA.BB and CC.AA had similar frequencies for autosyndesis, but AA.CC did not have this pairing; AA.CC and CC.AA showed similar frequencies for allosyndesis but higher than that in AA.BB. For the pairing within B genome, AA.BB and BB.CC had no autosyndesis but had similar low frequencies for allosyndesis. For the pairing with C genome, AA.CC, CC.AA, and BB.CC exhibited no autosyndesis, and AA.CC and CC.AA presented similar frequencies for allosyndesis but much higher than that in BB.CC. The higher frequency of allosyndesis in AA.CC and CC.AA than in AA.BB and BB.CC was attributable to the closer relationships between A and C genomes than with B genome.

Discussion

In this study, the chromosome pairings in the synthetic *Brassica* hybrids and allopolyploids with different genomic composition and cytoplasm were characterized by GISH and BAC-FISH, and the impacts of the hybrid genomic structure on the rates of auto- and allosyndesis and on the stability of the genomes at diploid stage were revealed. The level and frequency of auto- and allosyndesis for each genome varied significantly across hybrids, and those for A and C genomes were in wider ranges of variations than for B genome. The level of autosyndesis was lower than that of allosyndesis (Table 1 and Table 2; Figure 1 and Figure 3). The meiotic pairing in the synthesized allotetraploids was nondiploidized and was affected by the genome and cytoplasmic types (Table 3). The use of the same three parents in pair crosses eliminates the genotypic effect and makes the results in these hybrids comparable.

Genome structure of Brassica diploids

There has been a continuous debate and conflicting views on the origin and evolution of basic karyotypes in *Brassica*. The pachytene chromosome analysis of the basic genomes (Röbbelen 1960) provided compelling evidence in support of $x = 6$ as the constitution of the basic archetype. On the basis of marker arrangement conservation, Truco *et al.* (1996) also proposed a model of genome evolution that described these basic genomes as derived from six ancestral chromosomes that underwent several duplications and rearrangements. Recently, an ancestral Brassiceae karyotype (ABK) with six haploid chromosomes was proposed as the progenitor of tribe Brassiceae, which resulted from a reduction in chromosome number in ancestral crucifer karyotype (ACK, $n = 8$) or Proto-Calepineae karyotype ($n = 7$) (Lysak *et al.* 2006; Mandáková and Lysak 2008). This prototype (ABK) subsequently has diverged into *nigra*, *rapa*, and *oleracea* lineages 7.3–4 MYA (Wroblewski *et al.* 2000) or ~7.9 MYA (Lysak *et al.* 2005). $x = 6$ is most likely the basic chromosome number in the tribe Brassiceae and the genus *Brassica* (Prakash *et al.* 2011). Meiotic chromosome pairing in the haploids of *B. campestris* (syn. *B. rapa*, $2n = 10$, 3I + 2II + 1III) (Armstrong and Keller 1981), *B. oleracea* ($2n = 9$, 4I + 1II + 1III) (Thompson 1956; Armstrong and Keller 1982), *B. nigra* ($2n = 8$, 4 I + 2II) (Prakash 1973), and a related species, *B. tournefortii* ($2n = 10$, 3I + 2II + 1III) (Prakash 1974) also favored this proposal. Two A-A autosyndetic pairs and two C-C autosyndetic pairs were observed in haploids of *B. napus* cv. Darmor-bzh (Nicolas *et al.* 2007). In trigenomic interspecific hybrids (AABC, BBAC, and CCAB) from the crosses of *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC), a maximum of three A-A pairs, two B-B pairs, or two C-C pairs were observed across all hybrid types (Mason *et al.* 2010), although these genomes from natural allotetraploids have experienced the evolutionary process. A maximum of two B-B pairs appeared in trigenomic hybrids from crosses between natural and synthetic *B. napus* and *B. nigra* (AC.B, A.C.B) and between *B. carinata* and *B. rapa* (BC.A) (Ge and Li 2007), while the B genome was from allotetraploid *B. carinata* or diploid *B. nigra*. Our results showed that the maximum of two or three autosyndetic bivalents could occur in the A or C genomes in the haploid genomes of hybrids, but only two could occur in the B genome. The similar extents of autosyndesis within each genome from diploids and allotetraploids showed that the genome structure was largely maintained during allopolyploidization, but B genome was more stable than A or C genome. The consistent formation of two autosyndetic bivalents within B genome of diverse sources provided the chromosomal evidence for the proposal that the present *Brassica* genomes were derived from the basic karyotype with $x = 6$. Although the pairing is dependent on the structure of hybrids and on the presence or not of genetic control, no genetic factor for suppression of pairing was found in the B genome in trigenomic *Brassica* hybrids (Busso *et al.* 1987). The autosyndesis within one genome reflects the segmental homology between chromosomes caused by the rearrangements of the blocks or by the common origin of the chromosomes involved (Truco *et al.* 1996). Our present result also showed that the pairing in B genome was less affected by the genome and cytoplasm types. The recent draft genome sequence of *B. rapa* provided new data for the genome structure of *Brassica* diploids and revealed the almost complete triplication of the *B. rapa* genome relative to *Arabidopsis thaliana* and to ACK ($n = 8$) (Wang *et al.* 2011). But the triplication theory still fails to explain the origin of extant chromosome numbers.

Cytoplasmic and genomic effects on meiotic pairing in hybrids

Nuclear-cytoplasmic interactions are predicted to be important in allopolyploid and hybrid evolution (Gill 1991; Wendel 2000; Levin 2003) because the presence of a foreign nuclear genome in the cytoplasm from the female parent can result in nuclear-cytoplasmic incompatibilities. Chromosomal rearrangements in hybrids and allopolyploids could potentially occur in response to changes in nuclear-cytoplasmic interactions. Two types of cytoplasm exist in three *Brassica* diploids: the B type in *B. nigra* and the A/C type in *B. rapa* and *B. oleracea*. The A and B types are quite distinct although they retain homology to a large extent (Palmer *et al.* 1983; Yanagino *et al.* 1987; Warwick and Black 1991; Pradhan *et al.* 1992). The reciprocal synthetics of *B. juncea* (AABB/BBAA) showed directional genomic changes, with the significant alterations of the paternal genome. The reciprocal synthetic *B. napus* (AACC/CCAA) did not show different genomic changes. Song *et al.* (1995) suggested that this was attributable to the more closely related A and C cytoplasmic genomes and to the more compatible nuclear-cytoplasmic genomes in the AC and CA polyploids. Chromosome pairing studied in the large number of allohaploids produced from a wide range of *B. napus* accessions revealed two main clear-

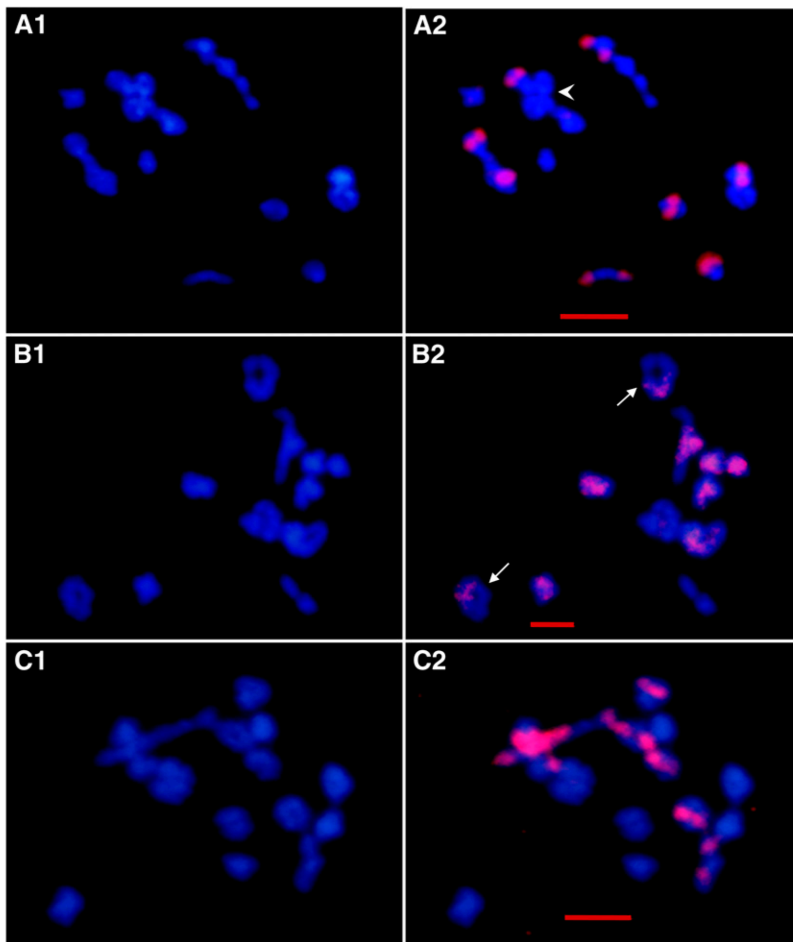

Figure 4 GISH/BAC-FISH analyses of meiotic chromosome pairings in synthetic *Brassica* allotetraploids. A1–C1 are DAPI (blue) images, and A2–C2 are merged images. (A1 and A2) One diakinesis of AA.BB with the following pairing: 10 II^{A-A} + 8 II^{B-B}. Arrowhead in A2 shows association of bivalents. Red signals are from *B. nigra*. (B1 and B2) One diakinesis of AA.CC with the following pairing: 8 II^{A-A} + 7 II^{C-C} + 2 $IV^{A-A-C-C}$ (solid arrow). Red signals are from the C-genome-specific BAC BoB014O06. Note that the two C-genome chromosomes labeled by the BAC in the two quadrivalents are homogenously and deeply stained by DAPI. (C1 and C2) One diakinesis of BB.CC with the pairing: 8 II^{B-B} + 9II^{C-C}. Red signals are from *B. nigra*. All bars, 5μm.

cut meiotic phenotypes that showed a twofold difference in the number of univalents at metaphase I and correlated with the only two plastid haplotypes identified in these accessions (Cifuentes *et al.* 2010). The segregation of two alleles at *PrBn* might explain a large part of the variation in meiotic behavior found among *B. napus* allohaploids. The results indicated that variation in crossover frequency among allohaploid genotypes generally correlates with the multiple origins of *B. napus* and *PrBn* diversity and also suggested the cytoplasmic and genetic effects on the meiotic crossover in allohaploids (Cifuentes *et al.* 2010). The significant reduction in crossover in all *B. napus* allohaploids compared with synthesized *B. oleracea* × *B. rapa* hybrids could reflect principally chromosome rearrangements that accentuated the divergence between *B. napus* homeologous chromosomes after the inception of this species. But the possibility that new nuclear-cytoplasmic interactions in new hybrids promote the crossover cannot be excluded.

The significant difference in chromosome pairing in the two hybrids A.C.B and C.A.B with the same nuclear genomes but a different cytoplasmic genome also showed the role of the cytoplasm in crossover frequency, although the A and C cytoplasmic genomes were closely related. The complex effects of the cytoplasm and nuclear genome interaction obviously resulted in the variation in crossover frequency of each genome among different hybrids (Table 1 and Table 2; Figure 3).

The trigenomic hybrids (AABC, BBAC, and CCAB) likely had the A/C type cytoplasm because they were produced from the crosses *B. juncea* × *B. napus*, *B. juncea* × *B. carinata* and *B. napus* × *B. carinata* (Mason *et al.* 2010). The comparison between the results from these hybrids and ours should reveal the effects of the cytoplasm and genome structure because both studies used the dual-color GISH and the same BAC clone (BoB014O06) from *B. oleracea*. Hybrids in the present study showed higher levels of autosyndetic pairing within haploid A and C genomes compared to the haploid B genome. Interestingly, the frequency of B-C pairs in AABC hybrids was much lower than in our B.C hybrid, but the frequency of A-C pairs in BBAC was higher than in our A.C hybrid, and also the frequency of A-B pairs in CCAB was lower than in our A.B hybrid. The frequency of A-B pairs in BBAC was a little lower than those in our BB.A, and A-C pairs in CCAB were nearly the same as those in our CC.A, but B-C pairs in CCAB were lower than in our CC.B. The different results from the two studies might be due to the presence of diversity in the genome structure in three diploids and allotetraploids of different origins or could be the effects of cytoplasmic genome. The allosyndetic bivalents in BB.A, CC.A, and CC.B were a little higher than in A.B, A.C,

Table 5 Means of chromosome numbers for auto- and allosyndesis within each genome in synthetic allotetraploids

	A genome		B genome		C genome	
Allotetraploids	Autosyndesis	Allosyndesis	Autosyndesis	Allosyndesis	Autosyndesis	Allosyndesis
AA.BB	0.14[a]	0.06[a]	0	0.06[a]	—	—
AA.CC	0	0.35[b]	—	—	0	0.35[b]
CC.AA	0.08[a]	0.23[b]	—	—	0	0.23[b]
BB.CC	—	—	0	0.03[a]	0	0.03[a]
Average	0.10	0.18	0	0.05	0	0.19

For the calculation of the total chromosome numbers involved in autosyndesis and allosyndesis within each genome in synthetic allotetraploids, A-A-C is taken as 1.5 A-genome chromosomes for autosyndesis and 0.5 A-genome chromosome and 1 C-genome chromosome for allosyndesis; A-A-A is taken as 1.5 for autosyndesis and 1.5 for allosyndesis; the homologous and homeologous ring quadrivalent (A-A-C-C) is divided into 1 A-genome and 1 C-genome chromosome for autosyndesis and 1 A-genome and 1 C-genome chromosome for allosyndesis; the homologous and homeologous ring quadrivalent (A-A-A-A) is divided into 2 equal A-genome chromosomes for homologous pairing and autosyndesis.
[a,b]Groups significantly different by χ^2 -test, $p < 0.05$.

and B.C, respectively, although only the difference between BB.A and A.B was significant (Table 1); the chromosome numbers for allosyndesis in CC.A and A.C showed only such a trend (Table 2). The information that the presence of a complete diploid genome enhanced homeologous pairing in trigenomic hybrids in comparison to allodiploids was also revealed in other *Brassica* hybrids (Nagpal *et al.* 1996), probably because digenomic triploids had more potential partners for homeologous pairing than allodiploids. Another reason might be related to the different ploidy levels, as meiotic recombination increases in *Arabidopsis* auto- and allopolyploids relative to diploids (Pecinka *et al.* 2011).

The genetic control of chromosome pairing in *Brassica* allopolyploids may be very complex. Even the major genetic factor *PrBn* that controls homeologous pairing at the haploid stage was detected (Jenczewski *et al.* 2003) and localized to linkage group C9 (Liu *et al.* 2006). But triploid AAC hybrids of the two *B. napus* genotypes for mapping *PrBn* as male with the same *B. rapa* variety displayed a similar meiotic behavior, which showed that *PrBn* had no effect on the meiotic behavior of triploid hybrids (Leflon *et al.* 2006) and that the gene was ineffective at hemizygous stage (Jenczewski and Alix 2004). But two AAC hybrids showed significant genotypic variation in crossover rates along a pair of A-genome chromosomes observed (Nicolas *et al.* 2009). The hybrids with Darmor-bzh of high pairing presented a reduction of autosyndesis within C genome, particularly of A-C allosyndesis compared with its haploid (Nicolas *et al.* 2007), showing that *PrBn* on the C genome failed to enhance or affect the pairing of its own chromosome in the AAC background. However, cytogenetic estimation of class I crossovers (interfering crossovers) in the entire genome by immunolocalization of a key protein, MutL Homolog1, showed that crossover rates were significantly higher in the allotetraploid AACC hybrid than in the diploid AA hybrid and were highest in the allotriploid AAC hybrid (Leflon *et al.* 2010).

Genome relatedness and chromosome behavior in synthetic allotetraploids

These synthetic *Brassica* allotetraploids showed substantial differences in their meiotic behaviors (Table 3 and Table 4). The associations between or among bivalents occurred in these allotetraploids with high frequencies (Figure 4), as observed in *Brassica* species previously (Maćkowiak and Heneen 1999). In the present study, AA.CC and CC.AA presented relatively high rates of univalents and the highest rate of homeologous pairing, as in other synthetic *B. napus* observed with the same BAC clone BoB014O06 from *B. oleracea* (Leflon *et al.* 2010). But AA.CC had high pollen fertility while CC.AA had much lower fertility, which was possibly caused by the cytoplasmic effects. The differences in cytological behaviors in our synthetics could be explained partly by their genome relatedness as revealed by the chromosome pairing in respective dihaploid hybrids from which they were derived (Tables 1–4) and as shown by other *Brassica* allopolyploids (Yao *et al.* 2012). The A-C bivalents in A.C were much higher than the A-B bivalents in A.B and the B-C bivalents in B.C. Hence, AA.CC formed A-C bivalents and A-A-C-C quadrivalents at a high rate, while AA.BB and BB.CC produced no homeologous bivalents except some homeologous quadrivalents (A-A-B-B or B-B-C-C) at low rates. Subsequently, AA.CC and CC.AA showed much higher frequencies for allosyndesis than AA.BB and BB.CC (Table 5). It was difficult to explain the much higher rate of univalents, particularly those of B genome in BB.CC than in AA.BB, because the cytoplasm from the B-genome donor in BB.CC should assist in stabilizing its chromosomes, while the B-genome chromosomes in the cytoplasm from the A-genome donor *B. rapa* in AA.BB was expected to show more aberrations.

The genetic changes caused by homeologous chromosome rearrangement were found to be common in newly resynthesized *B. napus* allotetraploids (Gaeta *et al.* 2007; Szadkowski *et al.* 2010, 2011). In the very first meiosis of synthetic *B. napus*, the frequent occurrence of A-C bivalents and/or multivalents and univalents was detected, which resulted in the production of gametes with unbalanced chromosomal composition and/or carrying chromosomal rearrangements (Szadkowski *et al.* 2010). The frequency of the meiotic-driven genetic changes depends significantly on the cytoplasm background inherited from the progenitors because the progenies of the synthetics with the *B. rapa* cytoplasm showed an excess of plants without rearrangements

and a lower frequency of plants carrying A1 marker loss than the one with *B. oleracea* cytoplasm. By contrast, no difference was found between C1 marker loss frequencies in the progenies of reciprocal synthetics. Conversely, the genetic backgrounds on *B. oleracea* cytoplasm did not influence the frequency of rearrangements. Furthermore, homeolog pairing and chromosome rearrangements, aneuploidy, and homeologous chromosome compensation were identified in 50 resynthesized *B. napus* lines across generations $S_{0:1}$–$S_{5:6}$ and in the $S_{10:11}$ generation by using a newly developed cytogenetic method to distinguish all 38 chromosomes in *B. napus* (Xiong *et al.* 2011). The data demonstrated that chromosome changes (aneuploidy and translocations) occurred most frequently on homeologous chromosome pairs that display the most extensive stretches of syntenic marker loci (Parkin *et al.* 2005; Udall *et al.* 2005; Gaeta *et al.* 2007; Nicolas *et al.* 2009). The two most unstable homeologous sets were A1/C1 and A2/C2, and their changes occurred in >50% of lines, including nullisomy, monosomy, trisomy, and tetrasomy because homeologous linkage groups A1/C1 and A2/C2 are each syntenic along their entire chromosome length (Parkin *et al.* 2005). Coincidently, two homeologous A-A-C-C quadrivalents formed in our synthetic *B. napus* (Figure 4B), which possibly involved the two groups A1/C1 and A2/C2. Accordingly, fewer chromosome changes were expected in synthetic *B. juncea* and *B. carinata* with more divergent genomes because their chromosomes showed a lower frequency of homeolog pairing (Table 4). Furthermore, the A, B, and C genomes showed different chromosomal stabilities in synthesized *Brassica* allohexaploids (Ge *et al.* 2009). Thus, it is worthwhile to trace the chromosomal rearrangements and stability across several generations in our synthesized allotetraploids in the future, especially in *B. juncea* and *B. carinata*.

Acknowledgments

We are grateful to Susan J. Armstrong and Steve L. Price (The University of Birmingham, Birmingham, UK) for providing the clone BoB014O06; to Genlou Sun (Saint Mary's University, Halifax, NS, Canada) for revising the manuscript; and to the two anonymous reviewers for their constructive comments. The study was supported by a special grant (Department of Science and Technology, Peoples' Republic of China) to National Key Laboratory of Crop Genetic Improvement.

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Communicating editor: J. C. Schimenti

GENETICS

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http://www.genetics.org/content/suppl/2012/04/13/genetics.112.140780.DC1

Cytoplasmic and Genomic Effects on Meiotic Pairing in *Brassica* Hybrids and Allotetraploids from Pair Crosses of Three Cultivated Diploids

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DOI: 10.1534/genetics.112.140780

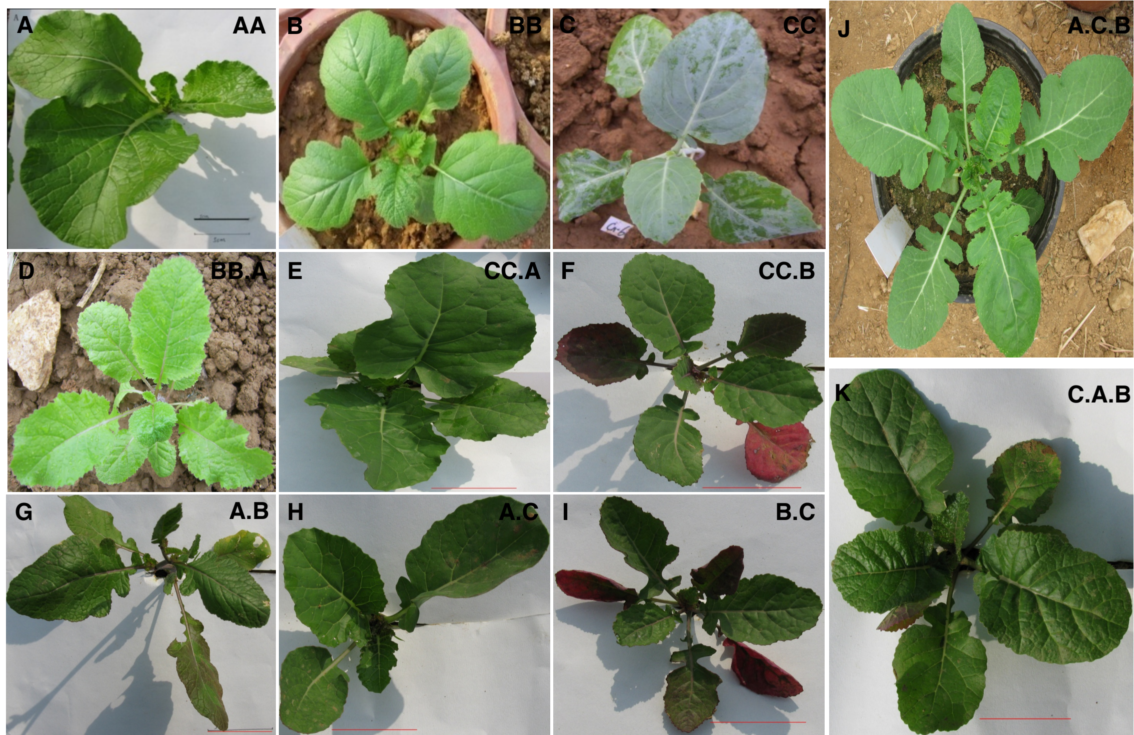

FIGURE S1 Morphology of young hybrids and their progenitors (A-K). (A) *B. rapa* (3H120), genomes AA. (B) *B. nigra* cv. Giebra, BB. (C) *B. oleracea* var. *alboglabra* L. (ChiJielan), CC. (D) BB.A. (E) CC.A. (F) CC.B. (G) A.B. (H) A.C. (I) B.C. (J) A.C.B. (K) C.A.B. All bars, 5cm

Table S1 Meiotic pairings in PMCs of hybrids at diakinesis and metaphase I by conventional method

Hybrids	Mean frequencies and Ranges						PMCs with M (%)	Total PMCs	Pollen fertility (%)
	I		Ⅱ		Ⅲ				
	Means	Ranges	Means	Ranges	Means	Ranges			
A.B	7.35	2-16	4.01	0-8	0.45	0-2	44.1	161	1.75
A.C	3.32	1-11	7.51	4-9	0.22	0-1	21.95	82	4.88
B.C	9.20	7-15	1.78	1-5	0.56	0-2	60.18	221	9.11
A.C.B	5.06	1-18	10.12	0-13	0.57	0-3	55.68	176	-
BB.A	7.62	4-12	8.40	2-10	0.50	0-2	45.28	84	25.33
C.A.B	5.99	1-11	9.74	0-12	0.51	0-1	50.83	120	-
CC.A	4.06	0-13	11.41	6-14	0.38	0-1	37.67	146	3.51
CC.B	6.49	4 - 13	8.51	2 - 10	0.83	0-3	56.6	53	17.56

Univalent = I, bivalent = Ⅱ, trivalent =Ⅲ, M: multivalents

Table S2   Means and percentages of trivalents per cell at metaphase I in hybrids revealed by FISH

Hybrids	Total range	A-A-A M (F%)	B-B-B M (F%)	C-C-C M (F%)	A-A-B M (F%)	A-B-B M (F%)	A-A-C M (F%)	A-C-C M (F%)	A-B-C M (F%)	B-B-C M (F%)	B-C-C M (F%)
A.B	0.75 0-3	0.03 (3.3)	-	-	0.44 (37.7)	0.28 (26.23)	-	-	-	-	-
A.C	0.86 0-3	0.23 (22.73)	-	0.10 (9.09)	-	-	0.32 (27.27)	0.23 (22.73)	-	-	-
B.C	0.65 0-3	-	0.03 (3.3)	0.10 (10)	-	-	-	-	-	0.25 (23.33)	0.27 (23.33)
A.C.B	0.41 0-1	-	-	-	-	0.05 (4.55)	0.14 (13.64)	0.05 (4.55)	0.18 (18.18)	-	-
BB.A	0.85 0-2	0.06 (5.88)	0.18 (17.65)	-	0.15 (14.71)	0.47 (38.24)	-	-	-	-	-
CC.A	0.44 0-1	-	-	0.12 (11.76)	-	-	0.06 (5.88)	0.26 (26.47)	-	-	-
CC.B	0.80 0-2	-	-	0.20 (10)	-	-	-	-	-	-	0.60 (50)

M: means of trivalents for each type. (F %): frequency of PMCs with trivalents.