Cytoplasmic and genomic effects on meiotic pairing in *Brassica* hybrids and allotetraploids from pair crosses of three cultivated diploids

Cheng Cui, Xianhong Ge*, Mayank Gautam, Lei Kang and Zaiyun Li*

National Key Laboratory of Crop Genetic Improvement, National Center of Crop Molecular Breeding Technology, National Center of Oil Crop Improvement (Wuhan), College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China.
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*Corresponding author: Zaiyun Li, Xianhong Ge, National Key Laboratory of Crop
Genetic Improvement, College of Plant Science and Technology, Huazhong
Agricultural University, Wuhan 430070, P. R. China.
Tel: 86-27-87281683
Fax: 86-27-87280009
E-mail: lizaiyun@mail.hzau.edu.cn, gexianhong@gmail.com
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 effects of the cytoplasm and genome combinations on the 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 pairing 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 B genome were less than that within A and C genomes, which had similar extent of pairing. Synthetic allotetraploids exhibited non-diploidized meiotic behavior, and their chromosomal instabilities were correlated with the relationship of the genomes and cytoplasmic background. Our results highlight the specific role of cytoplasm and genome to the chromosomal behaviors of hybrids and allopolyploids.

INTRODUCTION
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 (MASTERS 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 U-triangle (U 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 alloplid 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 allopolyploids (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* was suggested as potential plastid genome donor to *B. napus* (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 were 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 B genome has changed considerably; the B genome in *B. carinata* has unchanged but C genome considerably altered. In *B. napus*, both A and C genomes have undergone a similar extent of changes. Nevertheless, recent comparative sequence analysis between homoeologous 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 undergo changes than 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 homoeology 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 a major gene *PrBn* (Pairing regulator in *Brassica napus*) in *B. napus* was demonstrated to be responsible for inhibiting the homoeologous pairing in its haploids (JENCZEWSKI et al. 2003), which has been mapped on linkage group C9 and 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 homoeologous recombination regulation in the wild. The diploid-like meiotic behavior of allopolyploids is also thought to result from the divergence between homoeologous 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 homoeologous recombination in resynthesized *B. napus* is influenced by
their 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 non-diploidized behavior and was related to the genome affinity and cytoplasmic background. Our results provide a 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: *Brassica 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 about 20 days of pollination, the immature embryos were cultured on the 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 the MS medium with 1.5 mg L\(^{-1}\) 6-benzyl aminopurine (6-BA), 0.25 mg L\(^{-1}\)α-naphthalenacetic acid (NAA) to generate enough cloned plantlets for study. The cloned plantlets were cultured on MS agar medium with 1.5 mg L\(^{-1}\) 6-BA, 0.25 mg L\(^{-1}\) NAA and 100 mg L\(^{-1}\) colchicine for about 10 days to double chromosome for synthesizing the allotetraploids, 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 \textit{in vitro}.

\textbf{Cytological investigation and pollen fertility analysis:} The ovaries from young flower buds were collected and treated with 8-hydroxyquinoline for 3-4 hrs at room temperature before fixed in Carnoy’s solution I (3:1 ethanol: glacial acetic acid, \(v/v\)) and stored at \(-20^\circ\text{C}\) for further chromosome counting in somatic cells. The young flower buds were fixed directly in Carnoy’s solution I and stored at \(-20^\circ\text{C}\) 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.

**Probes and chromosome preparations:** The probes used for GISH and BAC-FISH were: 1) total genomic DNA of *B. nigra* cv. Giebra labeled with biotin-11-dUTP (Fermentas, China) by nick translation; 2) the plasmid DNA of BAC BoB014O06 (provided by Dr. Susan J. Armstrong, The University of Birmingham, UK) labeled with biotin-11-dCTP (for A.C and CC.A hybrids) by random priming using the BioPrime DNA Labeling System kit (Invitrogen, Life Technologies Corporation, USA) 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 Corporation, USA).

Total genomic DNA of *B. rapa* (3H120) and 45S rDNA was boiled for 15 min twice to obtain DNA fragments of 100 to 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 C0t-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, Japan), 0.2% pectinase (Merck, Darmstadt,
Germany), and 0.5% snailase (Beijing Baitai Biochem Co. Ltd., Beijing, China) 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, \(100 \mu g/ml\), 1 h at 37°C), then 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 and 0.5% SDS, 100 ng probes for each slide, while about 1000 ng blocking A genome DNA, \(1 \mu g\) Cot-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°C for 10 min. The probes and chromosomal DNA on the slides were co-denatured at 80°C for 5 min in a thermal cycler and hybridized at 37°C overnight in a humid chamber. Slides were washed stringently for 10 min in 0.1×SSC with 20% deionized formamide at 42°C. The immunodetection of biotinylated and digoxigenated DNA probe was carried out by using Cy3-labelled streptavidin (KPL, St. Louis, MO, USA) 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, Japan).
Images were processed by Adobe Photoshop (Adobe Systems, 15 San Jose, CA) to adjust contrast and brightness. Two-by-two chi-squared contingency tests were used to test the difference of pairing configuration.

**RESULTS**

**Morphology, cytology and pollen fertility in hybrids:** From reciprocal pair crosses of three *Brassica* diploids, the digenomic diploid and triploid hybrids were produced, A.B/BB.A, A.C/CC.A, 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 the reduced gametes by the male parent in the three crosses, though 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 a lot of 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 (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 those 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 with the labeled *B. nigra* genomic DNA probe, those in the A.C and CC.A hybrids by 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 centromeric region, therefore 45S rDNA and C0t-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 (Tables 1 and 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 A genome than B genome, for their rates of the univalents to the chromosome number of the genome were nearly the same. The maximum autosyndetic bivalent of A genome was three, one more than that of B genome, and the average of A genome (0.53) was higher than 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 PMCs, respectively (Table S2). The total average chromosomes per cell and the rates for autosyndesis within 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 of 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, 1.14, with maximum of three. The average (3.45) and maximum (7) of allosyndetic bivalents were much higher than those of autosyndesis (Table 1). The trivalents A-A-C and A-C-C were observed in 27.27% and 22.73% of PMCs, respectively (Table S2). The total chromosomes and rates for
autosyndesis were 3.50 and 0.35 in A genome, 2.91 and 0.32 for C genome, which were similar 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, 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, C genomes (0.64, 0.32) were much lower than for B genome (4.32). Inversely, the autosyndetic bivalents within A and C genomes (1.73, 1.14) were much higher than B genome (0.45). The average and maximum of allosyndetic bivalents were 4.50 and 8 for A.C, higher than 1.00 and 3 for A.B, 1.50 and 5 for B.C (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), 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.
Among the three digenomic diploid hybrids A.B, A.C, B.C, the average of bivalents in A.C was higher than in A.B and B.C. Such difference mainly resulted 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 (Tables 1 and 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 that in A.C, but autosyndesis for B genome was similar to A.B and B.C (Tables 1 and 2; Figure 3b, 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 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 and B.C, and those in A.C were higher than B.C. The values of B genome in A.C.B were similar to A.B or B.C, but the values in A.B were higher than 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 autosynthetic bivalents within A genome were 0.65 and 2, fewer than those of A-B allosynthetic bivalents (2.12 and 5). The average and maximum of homologous bivalents within B genome were 5.35 and 7, 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 2.68 ones for allosyndesis. Out of 16 chromosomes of B genome, 10.70 showed homologous pairing, 1.06 autosynthetic pairing, 2.68 allosynthetic pairing and 1.56 non-pairing (Table 2). In CC.A, the average and maximum of autosynthetic bivalents within A genome were 0.32 and 2, being fewer than those of A-C allosynthetic bivalents (3.76 and 8). The average and maximum of homologous bivalents within C genome were 6.29 and 9. 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 autosynthetic 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 (Tables 2 and 3); but the frequency of allosynthetic bivalents for A-B
(2.12) was lower than 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, which were lower than those of B-C allosyndetic bivalents (1.20 and 3). The average and maximum of homologous pairing within C genome were 6.70 and 8. 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). 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 chromosomes of 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 within B genome and B-C allosyndesis in two hybrids B.C and CC.B were similar (Tables 1 and 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, 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; Tables 1, 2 and 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 to the total chromosome number of the three genomes were similar in most hybrids, and only in A.C.B, the rates for auto- and allosyndesis within B genome were 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 C.C.B. The gross averages and rates for autosyndesis within each genome across all hybrids were also significantly lower than those of allosyndesis (Table 2). Though the average chromosomes for auto- and allosyndesis in B genome (1.19, 2.47) were fewer than 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, 0.26 and 0.32 for A, B, C genomes, respectively. Among the hybrids with haploid A genome (A.B, A.C, A.C.B, B.B.A and C.C.A), the chromosomes and rates of autosyndesis within A genome in A.C.B and A.C were much higher than B.B.A and A.B, and then higher than C.C.A, except the rates between A.B and B.B.A, B.B.A and C.C.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 chromosomes for autosyndesis within C genome in A.C were higher than B.C, not than A.C.B (Table 2), suggesting that the A and B genome 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 CC.A (0.59) was possibly caused by the less allosyndesis for more distant relationship between B and C genomes than 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 then higher than BB.A, and the rate in A.C.B was higher than that in the other four with no significant differences (Figure 1 and 3c; Table 1). This result showed that the introduction of B genome could enhance the A-C association, and also that the close homoeology existed between A and C.
genome, because the haploid or diploid C genome did not change their homoeologous 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, 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 homoeologous pairing of the two related genomes. The B-genome chromosomes for allosyndesis in B.C, 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 homoeologous 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, the univalents and multivalents appeared frequently (Table 3). The data from conventional and GISH observations (Tables 3 and 4) showed that the average of univalents in AA.BB was significantly lower than in other three, and that in
AA.CC and CC.AA was lower than 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 expectable, 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/CC.AA (83.89%/33.33%) were possibly attributable to the cytoplasmic effect, since their chromosome pairing behaviors were identical except the different rates of PMCs with multivalents (4.49%/11.68%) (Table 3).

With GISH/BAC-FISH analyses, the genome-specific homologous and homoeologous 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 C-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 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, not in AA.BB. The means of
C-genome homologous bivalents were similar among AA.CC, CC.AA and BB.CC.
The homoeologous bivalents and trivalents were only observed in AA.CC and CC.AA,
respectively. The quadrivalents involving the chromosomes of one or two genomes
were formed in these allotetraploids, and 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 pairing per cells 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 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 allosynedsis within B genome at the same
frequency as A genome. 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 had no 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 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 both with B genome.

**DISCUSSION**

In this study, the chromosome pairings in the synthetic *Brassica* hybrids and allopolyplploids 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 (Tables 1 and 2; Figures 1 and 3). The meiotic pairing in the synthesized allotetraploids was non-diploidized and was affected by the genome and cytoplasmic types (Table 3). The use of 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 basic archetype. On the basis of marker arrangement conservation, TRUCO *et al.* (1996) also proposed a model of genome evolution that these basic genomes were derived from six ancestral chromosomes that underwent several duplications and rearrangements. Recently, Ancestral Brassiceae Karyotype (ABK) with 6 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 (PCK, \( n = 7 \)) (LYSAK *et al.* 2006; MANDÁKOVÁ and LYSAK 2008). This prototype (ABK) subsequently has diverged into nigra, rapa and oleracea lineages during 7.3 - 4 Mya (WROBLEWSKI *et al.* 2000) or about 7.9 Mya (LYSAK *et al.* 2005). This \( x = 6 \) 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 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 allopolyplloidization, 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. Though 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 A. 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 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 on the large number of allohaploids produced from a wide range of *B. napus* accessions revealed two main clear-cut meiotic phenotypes which 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 principally reflect chromosome rearrangements that accentuated the divergence between *B. napus* homoeologous 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 different cytoplasmic genome also showed the role of the cytoplasm on crossover frequency, although the A and C cytoplasmic genome was 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 (Tables 1 and 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 and B. carinata, 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, since both studies used the dual-color GISH and the same BAC clone BoB014O06 from B. oleracea. Hybrids in 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 frequency of A-C pairs in BBAC was higher than in our A.C hybrid, and also frequency of A-B pairs in CCAB lower than in our A.B hybrid. A-B pairs in BBAC was little lower than those in our BB.A, 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 little higher than in A.B, A.C and B.C, respectively, although only the difference between BB.A and A.B was significant (Table 1), but the chromosome numbers for allosyndesis only in CC.A and A.C showed such trend (Table 2). The information that the presence of a complete diploid genome enhanced
homoeologous 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 homoeologous 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 homoeologous 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 behaviour, which showed that *PrBn* had no effect on the meiotic behaviour 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 cross-over 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 (Tables 3 and 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 present study, AA.CC and CC.AA presented relatively high rates of univalents and highest rate of homoeologous pairing, as in other synthetic *B. napus* observed with the same BAC clone BoB014006 from *B. oleracea* (LEFLON et al. 2010). But AA.CC had high pollen fertility while CC.AA had much low fertility, which was possibly caused by the cytoplasmic effects. The differences in cytological behaviors in our synthetics could partly be explained by their genome relatedness revealed by chromosome pairing in respective dihaploid hybrids from which they derived (Tables 1-4), as shown by other *Brassica* allopolyploids (YAO et al. 2012). The A-C bivalents in A.C were much higher than A-B bivalents in A.B and B-C bivalents in B.C. Hence, AA.CC formed A-C bivalents and A-A-C-C quadrivalents at high rate, while AA.BB and BB.CC produced no homoeologous bivalents except some homoeologous 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, for the cytoplasm from B-genome donor in BB.CC should assist in stabilizing its chromosomes, while the B-genome chromosomes in the cytoplasm from A-genome donor *B. rapa* in AA.BB was expected to show more aberrations.

The genetic changes caused by homoeologous 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, for 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, homoeolog pairing and chromosome rearrangements, aneuploidy, and homoeologous chromosome compensation were identified in 50 resynthesized *B. napus* lines across generations $S_{0:1}$ to $S_{5:6}$ 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 homoeologous
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 homoeologous sets were A1/C1 and A2/C2, and their
changes occurred in more than 50% of lines, including nullisomy, monosomy, trisomy,
and tetrasomy, since homoeologous linkage groups A1/C1 and A2/C2 are each
syntenic along their entire chromosome length (PARKIN *et al.* 2005). Coincidently,
two homoeologous A-A-C-C quadrivalents formed in our synthetic *B. napus* (Figure
4B), which possibly involved these 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 lower frequency of
homoeolog 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 future, especially *B. juncea* and *B.
carinata*.

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

<table>
<thead>
<tr>
<th>Hybrids</th>
<th>2n</th>
<th>Chromosome associations (Ranges)</th>
<th>PMCs with M PMCs</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>I A</td>
<td>I B</td>
<td>I C</td>
</tr>
<tr>
<td>A.B</td>
<td>18</td>
<td>8.26</td>
<td>4.74</td>
<td>3.53</td>
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<tr>
<td>A.C</td>
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<tr>
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<tr>
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<td>26</td>
<td>6.40</td>
<td>4.80</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Univalent = I, bivalent = II, trivalent = III, quadrivalent = IV. M: multivalent. 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, A.C.B hybrids, but $II^{B-B}$ and $II^{C-C}$ means homologous bivalents in BB.A, CC.A, CC.B hybrids; $II^{A-B}$, $II^{A-C}$ and $II^{B-C}$ indicate allosyndetic bivalents formed between A and B chromosomes, A and C chromosomes and B and C chromosomes, respectively. $^a$: Shared letters within each type of association denote that the values are insignificantly different (the chi-square test, Alpha=0.05)
TABLE 2 Means of chromosome numbers and ratios for no pairing (No), auto- and allosyndesis (Au, Al) within each genome in hybrids

<table>
<thead>
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<th>Hybrids</th>
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<th>B genome</th>
<th>C genome</th>
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<td>Al</td>
</tr>
<tr>
<td>A.B</td>
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<td>3.33&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>(0.19&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(0.33&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
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<tr>
<td></td>
<td>(0.26&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(0.35&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>(0.39&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
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<td>-</td>
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<td>(0.37&lt;sup&gt;c&lt;/sup&gt;)</td>
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<td>B.B.A</td>
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<td>1.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.57&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>(0.17&lt;sup&gt;ab&lt;/sup&gt;)</td>
<td>(0.27&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>C.C.A</td>
<td>5.18</td>
<td>0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(0.52&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>(0.07&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>(0.41&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>C.C.B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.60&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>(0.18&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>(0.23&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
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<td>Average</td>
<td>4.21</td>
<td>2.07</td>
<td>3.72</td>
</tr>
<tr>
<td></td>
<td>(0.42)</td>
<td>(0.21&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>(0.37&lt;sup&gt;a&lt;/sup&gt;)&lt;sup&gt;**&lt;/sup&gt;</td>
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For the calculation of the total chromosome number 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 genome, 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 3.5 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 auto-, allosyndesis and no pairing within each genome are the observed chromosome numbers/the total chromosome number 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. *: Shared letters within auto- and allosyndesis denote that the values are insignificantly different (the chi-square test, Alpha=0.05). The -, -- indicates that the averages of auto- or allosyndesis from different genomes in each hybrid are insignificantly different (the chi-square test, Alpha=0.05), respectively.
<table>
<thead>
<tr>
<th>Allotetraploids</th>
<th>2n</th>
<th>Means and ranges</th>
<th>Pollen fertility</th>
</tr>
</thead>
</table>
|                | I  | II  | III | IV | PMCs with multivalent PMCs | Total PMCs | (%)
|                | (Ranges) | (Ranges) | (Ranges) | (Ranges) | (Range) | (%) |
| AA.BB          | 36 | 0.04| 17.85 | - | 0.08 | 7.09 | 141 | 88.20 |
|                | (0-2) | (16-18) | - | (0-1) |
| AA.CC          | 38 | 0.45 | 18.64 | - | 0.07 | 4.49 | 89 | 83.89 |
|                | (0-10) | (14-19) | - | (0-2) |
| CC.AA          | 38 | 0.36 | 18.64 | 0.01 | 0.10 | 11.68 | 182 | 33.33 |
|                | (0-6) | (12-19) | (0-2) | (0-2) |
| BB.CC          | 34 | 0.73 | 16.62 | - | 0.01 | 0.73 | 137 | 60.52 |
|                | (0-6) | (14-17) | - | (0-1) |

Univalent = I, bivalent = II, trivalent = III, quadrivalent = IV. a: Shared letters within each association type denote that the values are insignificantly different (the chi-square test, Alpha=0.05)
<table>
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<tr>
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<th>Chromosome associations (Ranges)</th>
<th>Total</th>
<th>PMCs</th>
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<td>III</td>
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<td>17.74</td>
<td>9.80^b</td>
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<td>(8-10)</td>
<td>(7-8)</td>
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<td>0.35^b</td>
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<td>(0-10)</td>
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<td>(0-8)</td>
<td>(0-6)</td>
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<td>(0-4)</td>
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<td>(0-2)</td>
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Univalent = I, bivalent = II, trivalent = III, quadrivalent = IV. 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:

Shared letters within each association type denote that the values are insignificantly different (the chi-square test, Alpha=0.05)
TABLE 5 Means of chromosome numbers for auto- and allosyndesis (Au, Al) within each genome in synthetic allotetraploids

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<th>B genome</th>
<th>C genome</th>
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<td>Au</td>
<td>Al</td>
<td>Au</td>
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<td>0</td>
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<tr>
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<td>0</td>
<td>0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>CC.AA</td>
<td>0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>BB.CC</td>
<td>-</td>
<td>-</td>
<td>0</td>
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<td>0.18</td>
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For the calculation of the total chromosome number 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 homoeologous 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 homoeologous ring quadrivalent (A-A-A-A) is divided into equal 2 A-genome chromosomes for homologous pairing and autosyndesis. a: Shared letters within auto- and allosyndesis denote that the values are insignificantly different (the chi-square test, Alpha=0.05).
FIGURE 1. - Schematic illustrations 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), *B. oleracea* (CC genome). The digenomic diploid or triploid hybrids obtained are indicated between two parents, and the trigenomic triploids 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, those of autosyndetic bivalents are shown on the double lines connecting the same genome at the three joints.

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-A2) One diakinesis PMC from A.B with the pairing: 6 I^A^ + 4 I^B^ + 1 II^AA^ (open arrow) + 1 II^BB^ (solid arrow) + 2 II^AB^ (solid arrowhead). Red signals are from labeled *B. nigra* probe. (B1-B2) One diakinesis PMC from A.C with the pairing: 7 I^A^ + 6 I^C^ + 1 II^AA^ (open arrow) + 1 II^CC^ (arrow) + 1 II^AC^ (solid arrows with ball tail). Red signals are from C genome specific BAC BoB014O06. (C1-C2) One MI PMC from B.C with the pairing: 3 I^B^ + 4 I^C^ + 5 II^BC^ (solid arrow with ball tail). Red signals are from *B. nigra* probe. (D1-D2) One MI PMC from A.C.B with the pairing: 3 I^A^ + 4 I^B^ + 1 II^AA^ (open arrow) + 1 II^BB^ (solid arrow) + 2 II^CC^ (arrow) + 1 II^AB^ (solid arrowhead) + 4 II^AC^ (solid arrow with ball tail) + 1 II^BC^ (arrowhead). Red signals are from *B. nigra* probe, green signals are from C genome specific BAC BoB014O06. All bars, 5µm.

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 auto- and allosyndesis (Au, Al) within each genome across hybrids (c). Shared letters within each type denote that the values are not significantly different (the contingent chi-square tests, Alpha=0.05).

**FIGURE 4.** - GISH/BAC-FISH analyses of meiotic chromosome pairings in synthetic *Brassica* allotetraploids. A1-C1 are DAPI (blue) images, A2-C2 are merged images. (A1-A2) One diakinesis of AA.BB with the pairing: 10 \( II^{AA} + 8 II^{BB} \). Arrowhead shows association of bivalents. Red signals are from *B. nigra*. (B1-B2) One diakinesis of AA.CC with the pairing: 8 \( II^{AA} + 7 II^{CC} + 2 IV^{AA\cdotCC} \) (solid arrow). Red signals are from 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-C2) One diakinesis of BB.CC with the pairing: 8 \( II^{BB} + 9 II^{CC} \). Red signals are from *B. nigra*. All bars, 5\( \mu m \).
## Supplementary Material-Supporting Table

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Univalent = I, bivalent = II, trivalent = III, M: multivalents
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<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
<td>M (F%)</td>
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</tr>
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M: means of trivalents for each type. (F %): frequency of PMCs with trivalents.
Supplementary Material-Supporting Figure

**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. (Chülelan), 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