

Intra- and intergenomic relationships in interspecific hybrids between *Brassica* (*B. rapa*, *B. napus*) and a wild species *B. maurorum* as revealed by genomic in situ hybridization (GISH)

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Abstract Interspecific hybridization plays a crucial role in plant genetics and breeding. The efficiency of interspecific crosses to a considerable extent depends on the genetic relatedness of genomes from parental species. Interspecific hybrids involving *Brassica maurorum* ($2n = 16$, MM) and two *Brassica* crop species, viz *B. rapa* ($2n = 20$, AA) and *B. napus* ($2n = 38$, AACC), were produced and analyzed for their meiotic chromosome pairings in pollen mother cells (PMCs) by using genomic in situ hybridization (GISH) with the labeled DNA of *B. maurorum* (MM) as probe. In hybrids *B. maurorum* × *B. rapa* ($2n = 18$, MA), all chromosomes remained unpaired in 28% PMCs, and the maximum of autosyndetic bivalents was two and one among the chromosomes of A and M genomes, with the average per cell being 0.27 and 0.12, respectively. Up to two allosyndetic bivalents between A and M genomes appeared, averagely 0.48 per cell. In hybrids *B. maurorum* × *B. napus* ($2n = 27$, MAC), the maximum of autosyndetic bivalents in M genome was two and the average was 0.11, while the maximum of allosyndetic bivalents between M and A/C genomes was two and the

average was 0.78. The 2–7 bivalents formed by A/C-genome chromosomes showed their high homology. The results were compared and discussed with the chromosome pairings in the hybrids of *B. maurorum* with *B. juncea* and *B. carinata* with respect to the genome relationships and the potential for chromosome recombination.

Keywords *Brassica maurorum* · *Brassica rapa* · *Brassica napus* · Chromosome pairing · Genomic in situ hybridization (GISH) · Interspecific hybrid

Introduction

In spite of recent advances in applications of genetic engineering and molecular biology techniques for plant breeding, interspecific hybridization still plays a crucial role in crop improvement, by generating new ecotypes or species, broadening the genetic basis of crops and introducing agronomically important genes from wild species into crop germplasm. Furthermore, by investigating the homeologous chromosome pairing during the meiotic divisions in the wide hybrids, the genomic affinities and phylogenetic relationships of the diverse genomes could be established, which serve as a guide in successfully utilizing the alien germplasm resources.

The family *Brassicaceae* consists of a large number of wild and weedy species which are an

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excellent reservoir of useful genes for resistance to biotic and abiotic stresses. In *Brassica* crops, for their economic improvement, many interspecific/intergeneric hybrid combinations have been obtained, and in several instances, introgression of nuclear genes conferring desirable traits have been accomplished (see review by Prakash et al. 2009).

In this communication, we report the development of hybrids of *Brassica maurorum* with *B. rapa* and *B. napus* and their meiotic behaviour using GISH. *B. maurorum*, a related wild species, is reported to carry resistance to fungal diseases viz. white rust caused by *Albugo candida* and alternaria blight caused by *Alternaria* spp. (Chrungu et al. 1999). Although, genomic affinities based on chromosome pairing between *B. maurorum* and *B. rapa/B. napus* have been studied in the past (Takahata and Hinata 1983; Chrungu et al. 1999; Garg et al. 2007), the precise degrees of allo- or auto-syndetic pairings could not be ascertained as the parental chromosomes were undistinguishable by conventional cytological methods.

GISH not only discriminates the parental chromosomes in interspecific and intergeneric hybrids, but can also detect the chromosome recombinations (Stevenson et al. 1998; Kamstra et al. 1999; Karlov et al. 1999; Ji et al. 2004). GISH is also effective for identification of *Brassica* interspecific and intergeneric hybrids (Fahleson et al. 1997; Snowdon et al. 2000; Benabd-elmouna et al. 2003; Wang et al. 2005; Ge and Li 2007). In the present study, we applied the GISH technique to discriminate M genome from A/C genomes, and to visualize the extent of auto- and allosyndesis.

Materials and methods

Plant materials and crosses

The species used in the experiment were *B. rapa* L. ($2n = 20$, AA), *B. napus* L. ($2n = 38$, AACC) and *B. maurorum* Durieu ($2n = 16$, MM). *B. maurorum*, as the female parent, was pollinated by *B. rapa* (the combination designated as M.A) and *B. napus* (M.AC). The reciprocal cross *B. napus* \times *B. maurorum* (AC.M) was also made, but the hybrids were not analyzed for chromosome pairings by GISH. About 3 weeks after pollination, the immature embryos were rescued on MS (Murashige and Skoog 1962) agar medium without hormones. The plantlets from

embryo rescue were multiplied by continuously subculturing their axillary buds on MS medium with 1.5 mg/l 6-benzyl aminopurine (6-BA), 0.25 mg/l α -naphthalenacetic acid (NAA) to produce sufficient number of cloned plants, which were rooted and transferred to field for analyses.

For meiotic analysis, the young flower buds of F_1 plants were collected and fixed in 3 ethanol : 1 acetic acid solution (Carnoy's solution) overnight, transferred to the fresh solution and then stored in the refrigerator. For the chromosome counting in mitotic cells, the young ovaries were collected and then pretreated in 2 mM 8-hydroxyquinoline for 3 h and finally fixed in Carnoy's solution.

Probe labeling, chromosome preparation and GISH analyses

Total genomic DNA was extracted from young leaves by CTAB method. The DNA of *B. maurorum* was labeled with Bio-11-dUTP by nick translation and used as probe. The blocks were made by boiling the DNA of *B. rapa* and *B. napus* for 15 min.

Slide preparations of chromosomes for GISH were made mainly according to Zhong et al. (1996) and Ge and Li (2007) with some modifications. The enzyme mixture contained 0.6% cellulose "Onozuka" (Yakult Honsha, Japan), 0.4% pectinase (Merck, Germany) and 0.5% Snailase (Sabc, China).

In situ hybridization was carried out according to the protocol by Leitch et al. (1994). Hybridization signals of the *B. maurorum* probe were detected using Cy3-labeled streptavidin (Sigma, USA), and chromosomes were counterstained with 0.2% 4'-6-Diamidino-2-phenylindole (DAPI) solution (Roche, Basel, Switzerland), mounted in antifade solution (Vector) and examined under the fluorescent microscope (Nikon Eclipse 80i, Japan) equipped CCD camera. Images were processed by Adobe Photoshop (8.0).

Results

Hybridity in all the F_1 hybrids of the three combinations obtained following embryo rescue was confirmed cytologically, viz. $2n = 18$ for M.A and $2n = 27$ for M.AC and AC.M. and by intermediate morphology between the parents. The hybrid plants flowered abundantly, but were totally male and

Table 1 Chromosome associations in PMCs at diakinesis and metaphase I in hybrids M.A and M.AC

Hybrid	2n	Chromosome associations (means, ranges)								Total no. PMCs
		I _A	I _M	I _{A/C}	II _{A-A}	II _{A/C}	II _{M-M}	II _{A-M}	II _{M-A/C}	
M.A	18	8.97 (5–10)	7.28 (5–8)	–	0.27 (0–2)	–	0.12 (0–1)	0.48 (0–2)	–	172
M.AC	27	–	7.00 (3–8)	XX	–	XX (2–7)	0.11 (0–2)	–	0.78 (0–2)	123

XX data are not obtained

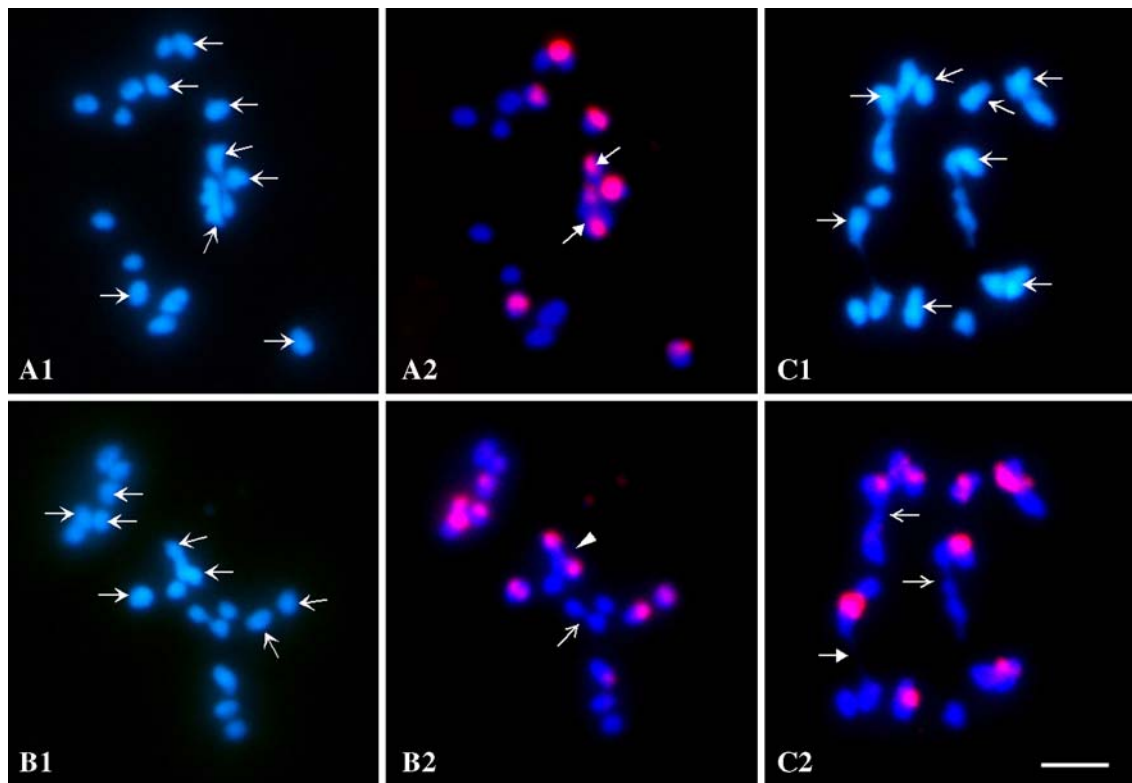


Fig. 1 GISH analyses of meiotic pairings in PMCs of hybrid *B. maurorum* × *B. rapa*. A1–C1, DAPI images (blue), M-genome chromosomes are marked by arrows. A2–C2 merged images, red signals are from *B. maurorum* probe. A2 Diakinesis

with two II_{AM} (solid arrow). B2 Diakinesis with one II_{AA} (line arrow) and one II_{MM} (arrowhead). C2 Diakinesis with two II_{AA} (line arrow), and one II_{AM} (solid arrow). Bar 5 μm

female sterile, and set no seed after selfing and open pollination.

By applying GISH with the labeled DNA of *B. maurorum* as probe, the chromosomes of M genome could be unequivocally distinguished from those of A and C genomes in pollen mother cells (PMCs) of M.A and M.AC at diakinesis/metaphase I, which made it possible to investigate the inter- and intra-genomic pairings. In M.A, 28% PMCs had all the chromosomes unpaired (18I) with an average of 8.97 univalents in A genome and 7.28 in M genome

(Table 1). Up to two bivalents were formed by A-genome chromosomes (Fig. 1 C2) with an average of 0.27 (Table 1), while a maximum of one bivalent with a mean of 0.12 was formed by M-genome chromosomes (Table 1, Fig. 1 B2). Allosyndetic pairing between A and M genome was noticed in 44% PMCs, with a maximum of two bivalents and an average of 0.48 bivalent (Table 1, Fig. 1 A2, C2).

In M.AC, the averages of univalents and autosyndetic bivalents were 7.0 and 0.11 within M-genome chromosomes, respectively (Table 1). The maximum

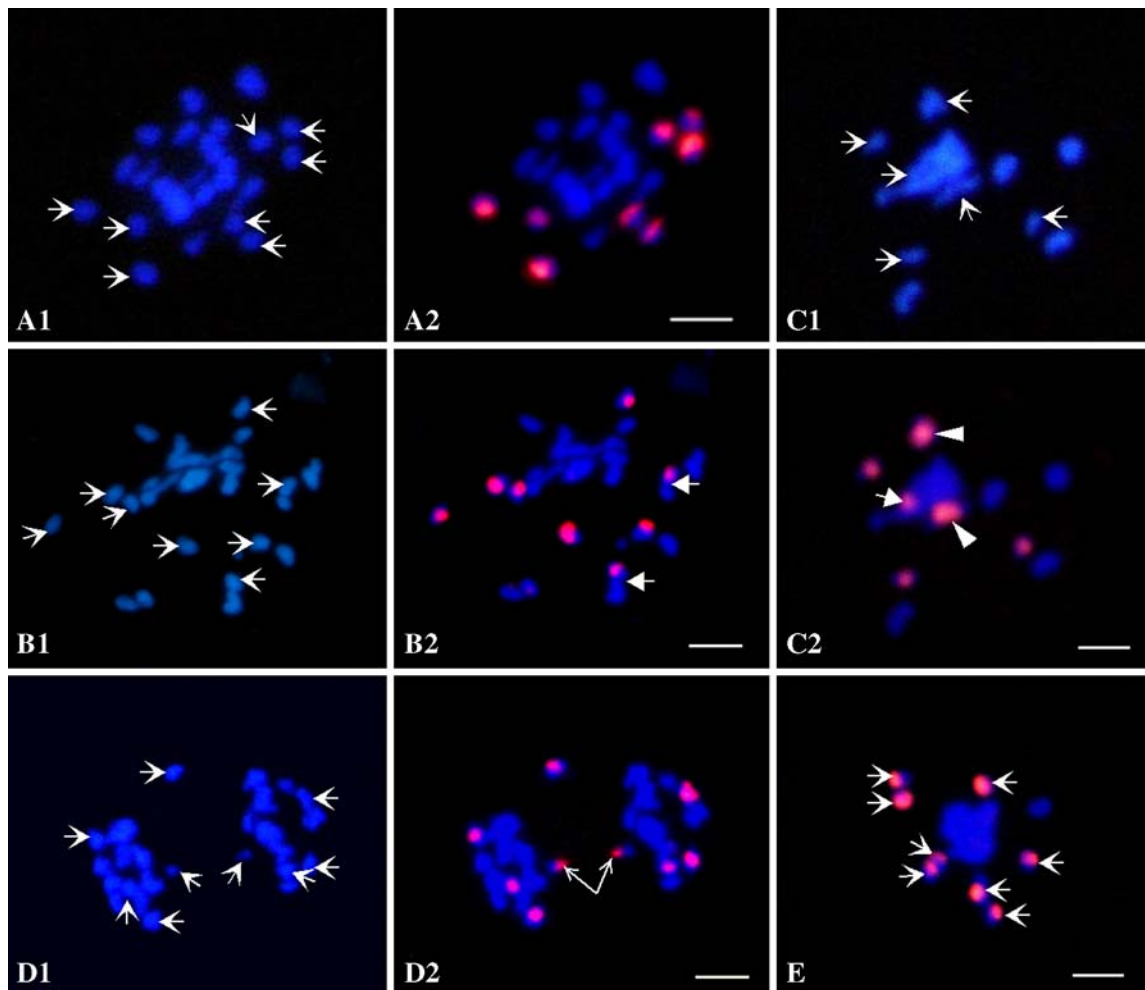


Fig. 2 GISH analyses of meiotic pairings in hybrid M.AC. *A1–D1*, DAPI images (blue), M-genome chromosomes are marked by arrows. *A2–D2*, *E* merged images, red signals are from *B. maurorum* probe. *A2* MI, seven bivalents formed between A/C-genome chromosomes, and M-genome chromosomes formed eight univalents. *B2* Diakinesis with two allosyndetic bivalents between M- and A/C-genome

chromosomes (solid arrow). *C2* MI with two II_{MM} (arrow-head) and one M-genome chromosome associated with one A/C-genome chromosome (solid arrow). *D2* AI with one M-genome chromosome lagged and another having sister chromatids separated (arrow). *E* MI with eight M-genome chromosomes (arrow) at the periphery. Bar 5 μ m

of autosyndetic bivalents was 2 (Table 1, Fig. 2 C2), higher than one in M.A. The mean of allosyndetic bivalents was 0.78 (Table 1), higher than 0.48 in M.A, while the maximal number (2) was the same as in M.A (Fig. 2 B2). All the PMCs observed showed at least two bivalents formed between A/C-genome chromosomes, even up to seven bivalents (Fig. 2 A2). In some PMCs at MI, the M-genome chromosomes were distributed around the periphery of the cells, while A/C chromosomes were at the center (Fig. 2 E). At anaphase I, individual M chromosomes

divided precociously (Fig. 2 D2) with a low frequency, but A/C chromosomes did not show such divisions.

Discussion

Because the ability to distinguish genomes from different species by GISH depends mainly on the degree of sequence homology (Ji et al. 2004), our results suggest that the sequence homology between

M and A/C genomes is relatively low. This conclusion is consistent with earlier cytological and molecular studies which assigned *B. maurorum* to the *nigra* lineage while *B. rapa* to a distant *rapa/oleracea* lineage (Takahata and Hinata 1983; Warwick and Black 1991; Pradhan et al. 1992). Detectable GISH signals on the chromosomes of M genome are mainly restricted to pericentromeric heterochromatin blocks where repetitive DNA sequences are clustered, as in other *Brassica* genomes (Snowdon et al. 1997; Ge and Li 2007). Due to small size of *Brassica* chromosomes, recombinant segments are difficult to detect effectively by GISH (Snowdon et al. 1997, 2000).

Since it is now conclusively established that *Brassica* genomes evolved from a common ancestral genome of $x = 3-7$ (see Prakash et al. 2009), chromosome pairing due to both auto- and allosyndesis is expected in hybrids of *Brassica* species. However, the frequency of allosyndesis between two genomes would reduce due to reproductive isolation, genetic mutation and regulation of chromosome pairing after the divergence of two genomes. In hybrids between crop brassicas and *B. maurorum* reported previously (Takahata and Hinata 1983; Chrungu et al. 1999; Garg et al. 2007), the chromosome associations investigated by conventional method were speculated to result from both auto- and allo-syndesis, but did not reflect the precise genomic affinity. Herein, with the extent of intra- and intergenomic pairings of M genome with A/C genomes determined by the method of GISH (Fig. 1, 2), the frequency of allosyndesis (0.48 A–M bivalent) in hybrid M.A is higher than that of autosyndesis (0.27, 0.12 bivalents within A and M genomes), reflecting the higher intergenomic homology and more chances for intergenomic recombinations. The average of allosyndetic bivalents (0.48) is also comparable to that (0.43 A–M bivalent) in the hybrid M.AB from the cross between the same *B. maurorum* type and *B. juncea* ($2n = 36$, AABB) but the maximal number (2) is less than that (3), where the degrees of all the types of auto-/allosyndetic pairs for three genomes are evaluated by dual GISH (Yao et al. 2010). The average of autosyndetic bivalents (0.27) within A genome in M.A is lower than 0.39 in M.AB, but the maximal number is the same, two. For the autosyndesis within M genome, the maximal number of bivalents is one for M.A and M.BC/BC.M from the reciprocal crosses of the same *B. maurorum*

type with *B. carinata* (Yao et al. 2010), and is two for M.AB and M.AC, while the average of bivalents is 0.26 for M.AB, higher than those in other four hybrids (0.11–0.14). One more autosyndetic bivalent in M.AC than M.A may be attributable to the preferential pairings of the chromosomes from A/C genome for their high homeology (up to seven bivalents formed), or the structural difference of A genome from *B. rapa* and *B. napus*.

Though A and C genome chromosomes distinction in *B. napus* has been recently realized by using DNA from *B. oleracea* as the probe and *B. rapa* DNA and the intergenic spacer of the *B. oleracea* 45S rDNA as the block (Howell et al. 2008), we failed to apply the procedure in the hybrid M.AC. Therefore, only the sum of allosyndetic pairings between A/C and M genomes are given, together with the autosyndetic pairing within M genome (Table 1). The average of 0.78 A/C–M bivalents in M.AC is nearly the same as the sums of C–M and B–M bivalents in M.BC/BC.M (0.70/0.68), but less the sum of A–M and B–M bivalents in M.AB (1.15), because the frequency for B–M (0.72) in M.AB was significantly higher than 0.39/0.37 in M.BC/BC.M. The average of A–B bivalent was higher than that of B–M and A–M in AB.M, and the average of B–C was higher than that for B–M or C–M in M.BC and BC.M. These results revealed a closer relationship between the basic genomes A and B or B and C than those between the three basic genomes and M. Furthermore, a maximum of three allosyndetic bivalents appeared between A–B, A–M, and B–M genomes in M.AB, respectively, but two were observed between B–C, B–M, and C–M genomes in M.BC and BC.M. The higher allosyndesis frequency of B–M than A–M or C–M in these hybrids also revealed their closer relationship (Takahata and Hinata 1983; Warwick and Black 1991; Pradhan et al. 1992).

Although the A and C chromosomes share very high homology (Truco et al. 1996) and pair preferentially in *B. napus* haploids (Nicolas et al. 2008) and triploid hybrids involving A/B/C genome (Ge and Li 2007), the chromosome homeology between M and A/C genome is high enough to lead to the formation of a maximum of two bivalents. Furthermore, the *B. napus* chromosomes in M.AC may have experienced high frequency of rearrangements, as detected in the *B. napus* haploid (Nicolas et al. 2007). So the structurally differentiated A/C chromosomes show

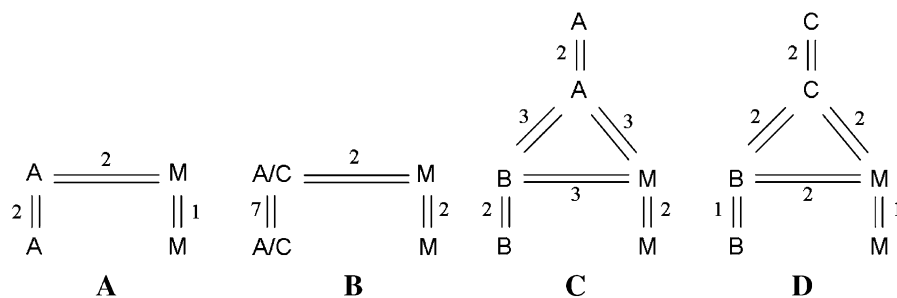


Fig. 3 The summary of autosyndesis and allosyndesis in hybrids of *B. maurorum* with *Brassica* species (*B. rapa*, A; *B. napus*, B; *B. juncea*, C; *B. carinata*, D). The maximum of auto- and allosyndetic bivalents is given for each type, and the

more allosyndesis, but the maximum of two allosyndetic bivalents between A/C and M genomes is lower than the expected five, for there are three A–M bivalents in M.AB, and two C–M bivalents in M.BC/BC.M (Fig. 3). Similarly, the significant difference in autosyndesis of the same M-genome in these hybrids (Fig. 3) could be due to the different genome combination or/and the structural differentiation of *Brassica* genomes from different origins, and the different chromosome numbers of three *Brassica* genomes ($n = 8, 9, 10$) will contribute to a different competitive condition to some extent.

The extent of autosyndesis in A, B, C genomes from three cultivated *Brassica* diploids was detected earlier from pairings in respective haploids. A maximum of two bivalents and one trivalent occurred in the haploid *B. campestris* (syn. *B. rapa*) (Armstrong and Keller 1981). The haploid *B. nigra* showed a maximum of two bivalents (Prakash 1973), *B. oleracea* haploid showed a maximum of two bivalents (Thompson 1956), or a maximum of one bivalent and one trivalent (Armstrong and Keller 1982). Two bivalents within B genome in trigeneric triploids ($2n = 27$, ABC) from *B. carinata* \times *B. rapa*, natural and synthetic *B. napus* \times *B. nigra* crosses were observed by GISH analysis (Li et al. 2005; Ge and Li 2007). These and present studies reveal the similar extent of autosyndesis in each *Brassica* genome from the extant diploids or allotetraploids (Fig. 3), suggesting that the main structures of these genomes are largely maintained during the evolutionary process of these allotetraploids after their formation by natural hybridizations between diploids, though genomic rearrangements are frequently detected in allopolyploids.

multivalents are not shown for very low number and frequency. The data from the hybrid M.BC are listed here, and those for the hybrid BC.M are nearly the same. The double lines represent bivalents

Extensive and diverse genomic changes can arise immediately at the onset of genome merging or within a few generations (Rieseberg et al. 1995; Song et al. 1995; Baack et al. 2005; Lukens et al. 2006). Homeologous exchanges have been shown to increase the range of genetic variation observed for important ecological and agronomic traits like flowering time (Pires et al. 2004) or seed yield (Osborn et al. 2003) in newly synthesized *B. napus*. The evidence presented here for chromosome association and chiasmata formation between M and A/C genomes demonstrates the feasibility of gene transfer from *B. maurorum* to *B. rapa* and *B. napus*. Therefore, exchange of chromosome fragments between M and A/C genomes via allosyndetic pairing is expected. The amphiploids originating from the *B. maurorum* \times *B. rapa* hybrid also showed resistance to *Alternaria brassicae* and *Albugo candida* (Garg et al. 2007), indicating the expression of resistant genes. To transfer the useful genes from M to A/C chromosomes, production of allohexaploids from the *B. napus* \times *B. maurorum* hybrids and backcrossing progenies with *B. napus* is being undertaken.

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