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ORIGINAL PAPER

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Reproduction and cytogenetic characterization of interspecific hybrids derived from crosses between *Brassica carinata* and *B. rapa*

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Abstract The tri-genomic hybrid (ABC, $2n=27$) between *Brassica carinata* (BBCC, $2n=34$) and *B. rapa* (AA, $2n=20$) is a unique material for studying genome relationships among *Brassica* species and a valuable bridge for transferring desirable characteristics from one species to the other within the genus *Brassica*. The crossability between *B. carinata* and *B. rapa* was varied with the cultivar of *B. rapa*. Hybrid pollen mother cells (PMCs), confirmed by morphological observation and molecular marker assay, could be grouped into 20 classes on the basis of chromosome pairing configurations. More than 30% of the PMCs had nine or more bivalents. Genomic in situ hybridization confirmed that two of the bivalents most likely belonged to the B genome. Nearly one-half of the PMCs had trivalents (0–2) and quadrivalents (0–2), which revealed partial homology among the A, B, and C genomes and suggested that there is a good possibility to transfer genes by means of recombination among the three genomes. The advantages of using the tri-genomic hybrids as bridge material for breeding new types of *B. napus* are discussed.

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Introduction

Brassica carinata (BBCC, $2n=34$) and *B. rapa* (AA, $2n=20$) have been cultivated for thousands of years (Gómez-Campo and Prakash 1999) as important oilseed crops. These two species are characterized by their high yield of seeds with a high oil content and by certain species-specific traits, such as self-incompatibility, earlier maturity, and disease resistance in *B. rapa* (Monteiro et al. 1988; Ren et al. 2000), and resistance against drought and pod shattering and a better performance under saline and late sowing conditions in *B. carinata* (Kumar et al. 1984; Malik 1990; Alonso et al. 1991). Interspecific hybridization between *Brassica* species has been an important means of creating new resources and combining valuable characters of different species, and it has been widely applied in plant breeding (Bing et al. 1991; Inomata 1997; Choudhary et al. 2000; Liu 2000). The ABC tri-genomic hybrid, derived from cross between *B. carinata* and *B. rapa*, has an important role in creating bridge materials for other *Brassica* species that have one or two of the three genomes. This hybrid is particularly valuable with respect to *B. napus* (AACC, $2n=38$), which is grown worldwide due to its superior seed yield and quality, specific characters that have only been available for about 400 years of its culturing history (Harlan 1971; Gómez-Campo and Prakash 1999). This paper reports the morphological characterizations as well as cytogenetic behavior of ABC hybrids between *B. carinata* and *B. rapa*.

Materials and methods

Plant materials and crosses

Three Chinese varieties of *Brassica rapa*, Shi (abbreviation from *Shiqian Baiyoucai*), Tian (from *Tianmen Youcaibai*), and Ya (from *Yaan Huang*), and one line of *B. carinata*, 10167, originally introduced from Ethiopia,

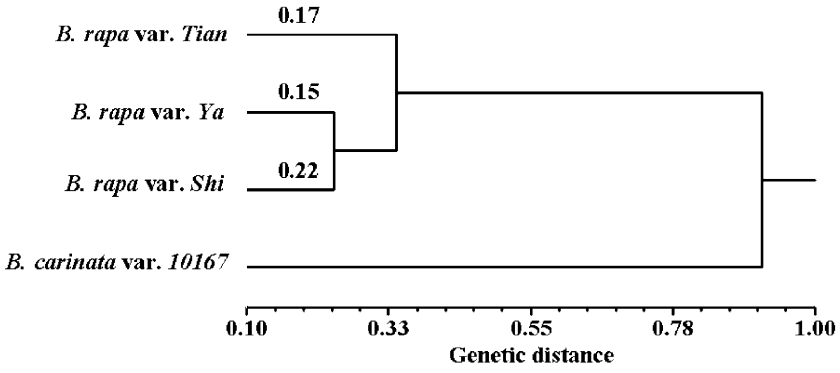

Fig. 1 Dendrogram of the genetic distances between three varieties of *Brassica rapa* and the *B. carinata* variety, 10167. The *number above the line* is the similarity coefficient between *B. carinata* and the variety of *B. rapa* linked to the *line*

were used to produce the hybrids. Reciprocal crosses between *B. carinata* and *B. rapa* were made by hand, and F_1 plants were grown in the field. The crossability between *B. carinata* and *B. rapa* was estimated based on the number of hybrid plants obtained from 100 cross-pollinated flowers.

Cytological analysis

Root tips from seeds or styles were used to determine the chromosome number of the hybrids. The materials were treated with 2 m *M* 8-hydroxyquinoline for 4–5 h at 22°C, fixed in Carnoy's solution for 24 h, and then stored in 70% ethanol at 4°C before chromosome observation (Li and Heneen 1999). The anthers were dissected out, cut in half, and stained with 10% modified carbol fuchsin for meiotic analysis (Li et al. 2001).

Total genomic DNA of *B. nigra* (BB, $2n = 16$) was extracted for GISH (genomic in situ hybridization) analysis according to the method of Horn and Rafalski (1992). DNA samples of the B genome were labeled with biotin following standard nick translation reactions according to the manufacturer (Sino-American Biology Co, Henan, P.R. China). GISH was performed according to the methods of Li et al. (2002,2004).

Pollen germination on the stigma and pollen-tube growth in the style were observed according to the method of Dumas and Knox (1983). Twenty-four hours following pollination, pistils were fixed in Carnoy's solution, treated with 8 *M* NaOH for 8 h, stained with 0.1% aniline blue solution, and examined with a fluorescence microscope.

Molecular marker analysis

Total genomic DNA was digested with *Eco*RI and *Mse*I for amplified fragment length polymorphism (AFLP) analysis. The adapter ligation and two successive PCR reactions were carried out according to the method described by Vos et al. (1995). Four pairs of primers with three selective nucleotides at the 3′ end were used: E^{+AGT}/M^{+CTC}, E^{+AAG}/M^{+CTC}, E^{+AGC}/M^{+CAA}, and E^{+AGC}/M^{+CAT}. Five primer pairs, Ra2-B01, Ra2-H10, Na10-A08, Na12-E09, and Na14-D09, downloaded from the *Brassica* database (http://www.ukcrop.net), were used for developing simple sequence repeat (SSR) markers. The PCR procedure followed was as described by Zhao and Meng (2003). The cluster analysis was done with NTSYS-PC VER. 2.1 software (Exeter Software, Setauket, N.Y.).

Results

Characterizations of the parents and hybrids

The genomic differences among the parents were estimated with 194 AFLP markers and 21 SSR markers. The molecular marker analysis showed that although the three cultivars of *B. rapa* were gathered into a single A-genome group, their genetic distance from *B. carinata* was different, with some being closer and some being more separated (Fig. 1). Eighty-four interspecific hybrid plants were obtained when *B. carinata* was used as the female parent, whereas only a few hybrids were produced in reciprocal crosses. Although the crossability varied greatly among the cultivars of *B. rapa*, there was no any evidence of a relationship between genetic distance and crossability (Table 1).

The hybrid plants were morphologically intermediate between the two parents. However, the stem at the position of the intergeniculum was always red, just as in the parent of *B. carinata*. Seventy-two of the hybrid plants had exactly 27 chromosomes, which is the chromosome number of the ABC-triploid somatic cells (Fig. 2a).

Table 1 Crossability of *Brassica carinata* × *B. rapa* with different male parents

Male parent	Number of flowers of *B. carinata* cross-pollinated	Number of hybrid plants obtained	Index of crossability
B. rapa var. Shi	156	15	9.6
B. rapa var. Tian	394	65	16.5
B. rapa var. Ya	84	4	4.8

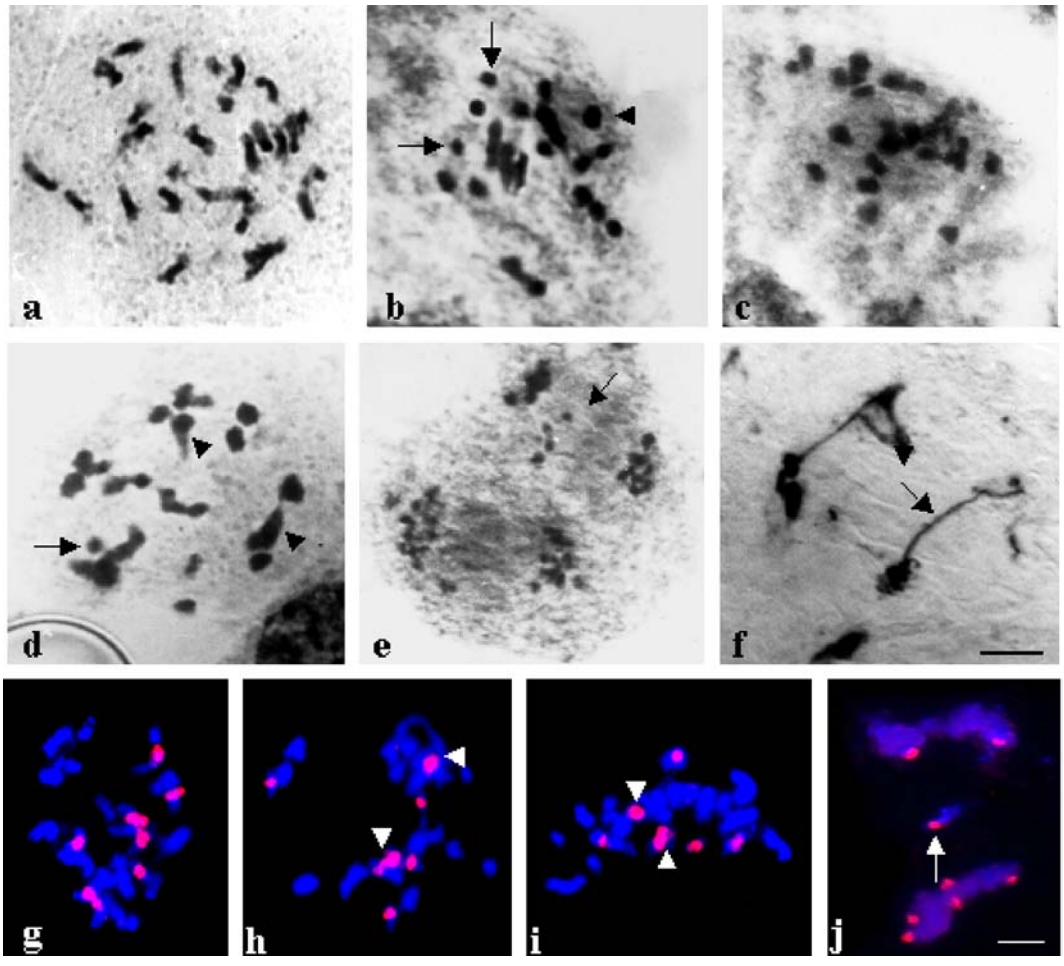

Fig. 2 Cytological characteristics of cells of triploid hybrid (ABC) plants derived from the cross of *B. carinata* var. 10167 × *B. rapa* var. Tian. Cells were stained with carbol fuchsin. **a** A cell from style tissue with the expected 27 chromosomes. **b** A typical chromosome association of 9 II + 9I (*arrow*), with the *arrow* and *arrowhead* showing the univalent and bivalent, respectively, at metaphase I (MI). **c** The chromosome association of 27 I at MI. **d** A PMC at diakinesis with univalents (*arrow*) and multivalents (*arrowheads*). **e** *Arrow* shows laggards of PMCs at anaphase II (AII). **f** *Arrow* shows one of two chromosome bridges at AII; cells were prepared with GISH. **g** A root tip cell with 27 chromosomes in which eight were hybridized with DNA of *B. nigra* (*red signal*). **h** A pollen mother cell (PMC) at diakinesis, two larger (*arrowheads*) and four small red signals were observed, indicating that two bivalents may have formed within the B genome. **I** A PMC at MI showing that two bivalents (*arrowheads*) formed between chromosomes of the B genome. **j** A PMC at AI showing that one laggard from the B genome stayed at the equatorial plate (*arrow*), while the other seven chromosomes of the B genome went to two poles with a 2:5 orientation. *Bar*: 10 μm

When the somatic cells of these hybrid plants were hybridized in situ with whole genomic DNA of *B. nigra*, eight chromosomes with strong signals could be clearly distinguished from the other 19 chromosomes (Fig. 2g). The interspecific hybrids were also characterized by SSR molecular markers in that the hybrids with 27 chromosomes possessed bands specific to both parents (Fig. 3).

The cytology of F_1 hybrids

Pollen mother cells (PMCs) from 35 hybrids involving two crosses, *B. carinata* var. 10167 × *B. rapa* var. Tian and *B. carinata* var. 10167 × *B. rapa* var Shi, were chosen randomly for further cytogenetic studies.

Chromosome pairing configurations in PMCs of the hybrids were very complex. From the 932 cells that were counted, there were an average of 10.31 univalents, 7.22 bivalents, 0.55 trivalents, and 0.11 quadrivalents in a cell, and 20 different combinations could be summarized: 4–27 univalents + 0–11 bivalents + 0–2 trivalents + 0–2 quadrivalents (Table 2). The most frequent configuration was 9II + 9I, which accounted for 25% of the cells observed (Fig. 2b). The strictly 27-univalent cells formed only a very small portion of the total (Fig. 2c). Conversely, there could be as many as 11 bivalents in a cell. Significant differences in the number of bivalents were found between crosses with two different cultivars of *B. rapa*, Tian and Shi: 68% of the PMCs of the Tian-derived hybrid had seven or more bivalents, while 82% of the PMCs of the Shi-derived hybrid had seven or more bivalents. One-half of the observed cells had at least one multivalent association in the form of trivalent or quadrivalent (Fig. 2d). The high

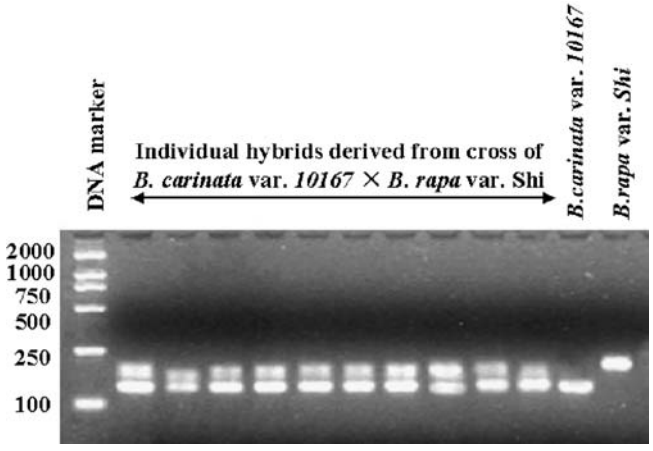

Fig. 3 Identification of interspecific hybrids between *B. carinata* and *B. rapa* with SSR markers amplified with the primer pair Na10-A08

Table 2 Statistics of chromosome associations at diakinesis/metaphase I in pollen mother cells (PMCs) of triploid hybrids

Class of PMC	Percentage of PMCs	State of chromosomes association			
		Univalent	Bivalent	Trivalent	Quadrivalent
1	24.79	9	9		
2	14.16	10	7	1	
3	9.33	8	8	1	
4	8.80	11	8		
5	6.87	6	9	1	
6	5.79	8	5	3	
7	4.83	21	1		1
8	3.86	13	7		
9	3.76	12	6		
10	3.00	5	11		
11	2.68	11	6		1
12	2.58	19	4		
13	2.36	7	7	2	
14	2.25	15	6		
15	1.29	4	7		2
16	1.07	10	5	1	1
17	0.97	11	5	2	
18	0.75	27	0		
19	0.54	25	1		
20	0.32	23	2		

frequency of bivalents and multivalents in PMCs indicated that both autosyndesis as well as allosyndesis must have occurred in the ABC tri-genomic hybrid. Some of the bivalents, two in most cases, should have come from the B genome, which consists of eight chromosomes, since two larger red signals with four small individual red signals were frequently observed with GISH at diakinesis and metaphase I (Fig. 2h, I)

A total of 372 PMCs of the hybrids were observed at anaphase I (AI) and anaphase II (AII). Lagging chromosomes appeared in profusion at both stages (Fig. 2e). GISH analysis revealed that the laggards belonged to the B or A/C genome (Fig. 2j). Although the majority of PMCs had laggards at AI/II, 11% of cells were laggard-free. A chromosome bridge was frequently observed at AI and AII in about 15% of the cells (Fig. 2f), indicating that crossovers occurred between homoeologous chromosomes or from 'accidents' with the lagging univalent.

The fertility of the hybrids

Consistent with the various meiotic irregularities that appeared in PMCs, the fertility of the pollen grains was very low and seed set was also very poor in the triploid plants compared with that of the parental cultivar of *B. carinata* and of *B. rapa* (Table 3). Seed set was occasionally achieved as a result of self-pollination or open pollination in the hybrid plants despite the absence of seed set in most cases. The pollen germination ratio and the seed number per siliquae were higher in hybrids with the *B. rapa* var. Shi than in hybrids involving *B. rapa* var. Tian, which was in accordance with the genetic relationship of these three cultivars. It would appear that the A genome in Shi was more compatible with the C genome of *B. carinata* when they coexisted within a cell.

Discussion

The genus *Brassica* consists of three elementary species, i.e. *B. rapa* (AA, $2n=20$), *B. oleracea* (CC, $2n=18$), and *B. nigra* (BB, $2n=16$). These three diploid species represent ascending aneuploids and are regarded as secondary balanced polyploids (Manton 1932). Recent

Table 3 Fertility of the triploid hybrids and their parental variety of *B. carinata* and *B. rapa*

Characters	Triploid hybrid		Parental cultivar		
	B. carinata var. 10167 × *B. rapa* var. Tian	*B. carinata* var. 10167 × *B. rapa* var. Shi	*B. carinata* var. 10167	*B. rapa* var. Tian	*B. rapa* var. Shi
Pollen germination ratio (%)[a]	4.4 (0.98)[d]	7.6 (1.24)	90.7 (2.52)	94.6 (1.78)	93.8 (1.38)
Length of siliquae (cm)[b]	1.73 (0.54)	1.96 (0.46)	5.22 (0.62)	7.51 (0.69)	7.87 (0.57)
Seeds per siliquae[c]	0.62 (0.16)	0.86 (0.14)	13.4 (0.31)	22.3 (0.78)	21.6 (0.69)

[a]Data collected from 250 pollen grains on stigmas
[b, c]Data measured with 20 siliquae
[d]Number in the bracket represent the standard deviation.

investigations on nuclear, mitochondrial, and chloroplast DNA restriction fragment length polymorphism (RFLP) established that *B. nigra* and *B. carinata* belong to the "*nigra*" lineage in contrast with the "*rapa*/*oleracea*" lineage that includes *B. napus* (Palmer 1988; Warwich and Black 1991; Gómez-Campo and Prakash 1999). GISH studies on *B. napus*, *B. juncea*, and *B. carinata* have also shown such lineage divergence as the B-genome chromosomes could be reliably distinguished from chromosomes of the A and C genomes (Snowdon et al. 1997; Li et al. 2002, 2004). In the present study, eight strong signals were detected when the DNA probe of *B. nigra* was hybridized with the chromosomes of the ABC tri-genomic hybrids. This result suggested that the chromosomes of the B genome have a low homology with those of the A and C genomes and that the B genome in the diploid of *B. nigra* is very conservative with respect to that of the alloploid of *B. carinata*.

The present study revealed a higher order of chromosome pairing, including 1–2 quadrivalents, 1–3 trivalents, and 1–11 bivalents, in the tri-genomic triploid hybrids (ABC, $2n = 27$), which indicates that autosyndesis as well as allosyndesis pairing have occurred. Attia and Röbbelen (1986) pointed out that seven bivalents on average were almost formed in the amphihaploid *Brassicas* with the AC genome. Prakash (1973) recorded a maximum of two bivalents resulting from autosyndesis in the haploid of *B. nigra*. Consequently, one might expect nine bivalents in the PMCs of the ABC hybrids. It is very interesting that nine bivalents formed in more than 30% of the PMCs in present study and that two of these most likely came from the B genome since GISH verified two larger red signals (bivalents) from other four smaller signals (univalent). As up to 11 bivalents could be formed in the ABC hybrid, some chromosome(s) from the B genome must be involved in pairing with the chromosomes of the A and/or C genome. The partial homology of the three genomes was also verified by the formation of trivalent and quadrivalent configurations, which is in agreement with previous observations (Mizushima 1950; Prakash and Hinata 1980; Prakash et al. 1999; Choudhary et al. 2000). The partial pairing of the B genome with the A/C genome hints at the possibility of transferring valuable traits from the B genome into the A/C genome. We self-pollinated the AABCC pentaploid, which was derived from crosses between the hexaploid (AABBCC) and *B. napus* (AACC), and obtained *B. napus* with 38 chromosomes. This material can be considered to be a new-type *B. napus* because not only did part of the A genome came from *B. rapa* and part of the C genome from *B. carinata*, but also some of the genetic composition of the B genome may have been transferred into the A/C chromosomes (Li et al. 2004). The breeding process of the new-type *B. napus*, and a molecular marker analysis will be reported in another paper.

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