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Production and genetic analyses of novel *Brassica rapa* L. introgressions from interspecific crosses with *Brassica juncea* L. landraces native to the Qinghai-Tibet Plateau

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Abstract Interspecific hybrids between related species have long been used for transferring desirable genes, broadening genetic diversity and utilizing intersubgenomic heterosis. In this study, we developed a novel *Brassica rapa* type (AA, 2n = 20) exhibiting certain features derived from interspecific hybridization between natural *B. rapa* and *Brassica juncea* (AABB, 2n = 36). In pollen mother cells (PMCs) of the novel *B. rapa* type, normal chromosome pairing with 10 bivalents and 10:10 segregation was observed, and the novel *B. rapa* lines were completely fertile. However, GISH showed that certain B chromosomes or fragments were introgressed into *B. rapa.* Genetic components of the novel *B. rapa* lines were investigated by GISH, AFLP and SSR analyses. GISH analysis of F_1, BC_1F_1, and BC_1F_2 plants confirmed the identities of three addition lines and seven translocation lines. AFLP and SSR analyses of 60 hybrid progenies from BC_1F_4 plants, their parents, and some *B. juncea* and *B. rapa* resources indicated that the A^J and B chromosome(s) or fragment(s) introgressed to the novel *B. rapa*. AFLP revealed that 60 BC_1F_4 plants contained B chromosomes or fragments, which evidenced introgression into the hybrid progeny. SSR analysis indicated that the A-genome (A1–A10) of *B. juncea* was introgressed into the hybrid progeny at 1.0 to 42.7%. Lastly, we obtained some yellow-seed and early-flowering *B. rapa* resources. The novel *B. rapa* lines can be used to genetically improve *B. rapa* in the Qinghai-Tibet Plateau and to study the origin and evolution of the A- and B-genomes.

Keywords Amplified fragment length polymorphism (AFLP) · *B. juncea* · *B. rapa* · Genetic introgression · Genomic in situ hybridization (GISH) · Simple sequence repeat (SSR)

Introduction

Introgressive hybridization plays a crucial role in the evolution of plant species (Ma et al. 2006) and is an important approach used to broaden the genetic base

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and transfer superior traits; it has been widely used for improving crops. Introgressive hybridization consists of incorporation of the genetic material from one species into another using distant hybridization and repeated backcrosses. The term "U triangle" refers to six agriculturally important *Brassica* species, three of which are diploids and the other three allopolyploids. Nagaharu (1935) showed that *B. rapa* (genome AA, 2n = 20) can synthesize two types of allopolyploids, with *B. nigra* (BB, 2n = 16) to form *B. juncea* (AABB, 2n = 36) and with *B. oleracea* (CC, 2n = 18) to form *B. napus* (AACC, 2n = 38); the other diploid, *B. nigra*, and *B. oleracea* form *B. carinata* (BBCC, 2n = 34). Plants with the B-genome, namely, *B. nigra* (BB, 2n = 16), *B. carinata* (BBCC, 2n = 34) and *B. juncea* (AABB, 2n = 36), have excellent resistance characteristics, e.g., heat and drought tolerance, seed shattering, and blackleg, that are effective throughout the life of the plant (Woods et al. 1991; Fredua-Agyeman et al. 2014). Therefore, to produce *B. rapa* or *B. napus* cultivars that have similar resistance characteristics, many researchers have attempted to use the B-genome from *Brassica* species as a donor in either sexual crosses (Zhu et al. 1993; Navabi et al. 2010, 2011) or somatic hybridization (Waara and Glimelius 1995).

Genomic in situ hybridization (GISH) is a sophisticated tool that has been successfully used to discriminate between parental genomes in interspecific introgression lines (Wang et al. 2004; Hua and Li 2006; Ma et al. 2006). GISH techinque utilizes total genomic DNA with specific probes that are easier to identify and amplify. In addition, the ratio of probe/blocking DNA should be sufficient to inhibit chromosome labeling of both genomes together, and blocking DNA plays an important role in hybrids derived from closely related species because there is high possibility that homology can be arisen during hybrid production (Xie et al. 2010; Younis et al. 2015). GISH offers direct visual analysis to differentiate parental genomes and can be used to investigate genome associations in allopolyploid species, interspecific introgression lines, and interspecific hybrids (Jiang and Gill 1994). GISH can also effectively and accurately identify alien chromatin stages and consolidation positions. Moreover, GISH has been successfully used to distinguish the parental chromosomes in intergeneric hybrids and interspecific hybrids with relatives and progenies, i.e., *Brassica* species (Ma et al. 2006; Kang et al. 2014; Yao et al. 2010), *Triticum* species (Molnár and Molnár-Láng 2010; Tang et al. 2014; Li et al. 2015), *Zea* species (Gonzalez et al. 2006), *Allium* species (Yamashita et al. 2005), orange (Fu et al. 2004), *Secale* species (Zhou et al. 2010) and *Lilium* species (Barba-Gonzalez et al. 2006).

Molecular markers have widely used to detect introgressive genes in *Brassica* species. Recently, a complete set of monosomic alien addition lines between *B. napus* and *Isatis indigotica* was developed using GISH/FISH and simple sequence repeat (SSR) markers (Kang et al. 2014). Intertribal somatic hybrids between *B. napus* and *Isatis indigotica* and backcross progenies were analyzed using GISH and amplified fragment length polymorphism (AFLP) markers (Du et al. 2009). The combination of GISH and molecular marker techniques can better characterize hybrids and their genomic changes, particularly karyotypically unstable or partial hybrids (Hua et al. 2006; Ma et al. 2006).

B. juncea has several valuable agronomic characteristics, including yellow seeds, high oil content, heat and drought tolerance, disease resistance, tolerance to poor soil, seed shattering resistance and early maturity (Woods et al. 1991; Dhaka et al. 2017). Some valuable characteristics of *B. juncea* introgressed into *B. rapa*, making it highly suitable for planting in spring rapeseed regions. In this study, to obtain novel *B.rapa*, some genes or fragments of *B. juncea* introgressed into *B. rapa* by interspecific hybridization, and the genomic compositions of novel *B. rapa* types were analyzed using GISH, AFLP and SSR. We finally obtained several novel *B. rapa* lines, i.e., yellow seeds and early flowering.

Materials and methods

Plant materials

The *B. juncea* cultivar "Luhuo" originating from Luhuo County, Sichuan Province, China, was used as the recipient parent, and the elite *B. rapa* landrace "Menyuan rapeseed" originating from the Qinghai–Tibetan plateau was used as the donor parent. All plants were self-pollinated for more than seven generations. The F_1 plants between *B. juncea* cv. Luhuo (2n = 4x = 36, AABB) and *B. rapa* cv. Menyuan (2n = 2x = 20, AA) were backcrossed to

Menyuan rapeseed to obtain BC_1F_1 populations and BC_1F_1 plants were self-pollinated to obtain BC_1F_2 plants, which were self-pollinated to obtain BC_1F_3 plants, which were selfed to obtain BC_1F_4 plants (Fig. 1). In addition, two parents, five contrast varieties and 60 BC_1F_4 plants were analyzed using molecular markers, together with Menyuan rapeseed, Luhuo, *B. nigra*, Haoyou 11 (landrace, *B. rapa*), Qingyou241 (*B. rapa*), Huangzi (*B. rapa*), Dahuang (landrace, *B. rapa*) and 60 hybrid progenies.

Observation of pollen viability

Anthers with mature pollen at the flowering stage were collected from 9 to 11 am, and pollen fertility was determined as the percentage of pollen grains stained with 1% acetocarmine. The stained pollen grains were observed under a fluorescence microscope (Nikon Eclipse 80i, Japan), and pictures were obtained with a Nikon DS-Fil camera.

Cytology observation

The ovaries and anthers from young flower buds were collected from 9 to 11 in the morning to determine the chromosome numbers and analyze the meiosis of hybrids and progenies. The ovaries were first pretreated with a 2 mM 8-hydroxyquinoline solution for 3 h at room temperature, fixed in Carnoy's solution (3:1, ethanol:glacial acetic acid, v/v) for 24 h and stored in 75% ethanol at − 20 °C until further use. The anthers were fixed in Carnoy's solution for 24 h and then stored in 75% ethanol at − 20 °C until further use. Cytogenetic observations were performed according to methods described by Li et al. (1995).

DNA isolation and GISH analysis

Genomic DNA was extracted using methods described by Warude et al. (2003), with some modifications. The DNA concentration and purity were measured at

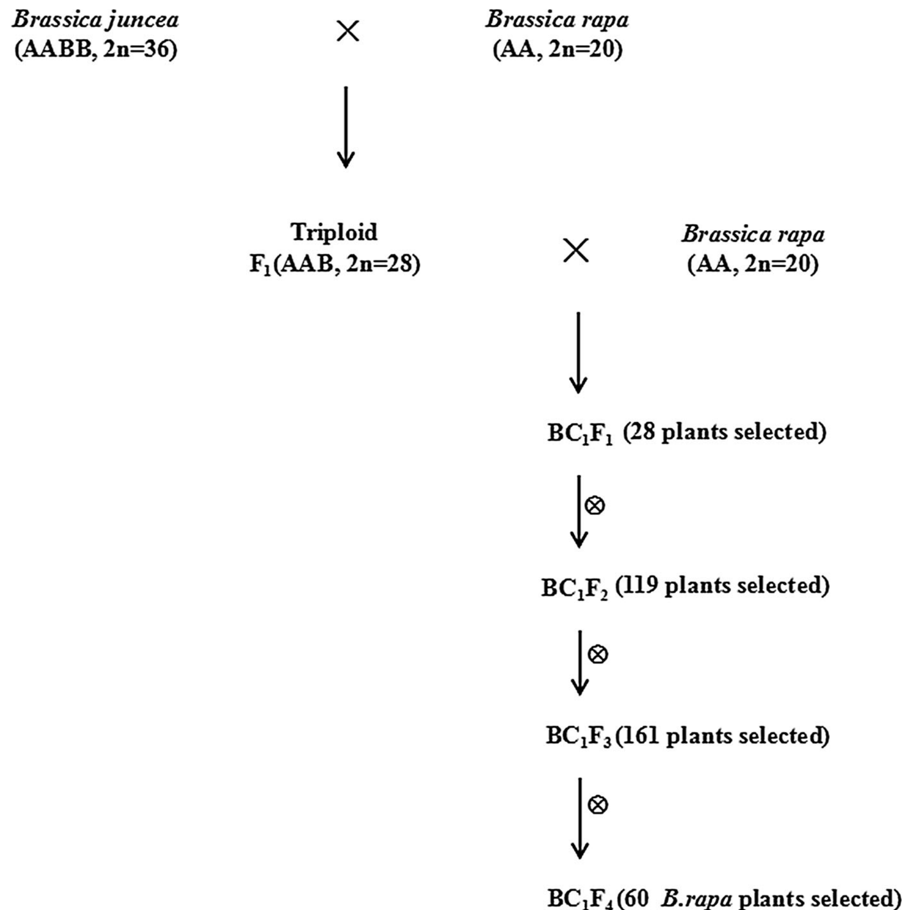

Fig. 1 Breeding diagram for developing of novel *B. rapa*

wavelengths of 260 and 280 nm, and the final DNA concentration was 50 ng/μl in TE buffer, which was suitable for AFLP and SSR analyses.

Total genomic DNA was isolated from the young leaves of *B. nigra* using the cetyltrimethyl ammonium bromide (CTAB) method. Slide preparations for GISH were generated according to the procedures described by Zhong et al. (1996). The *B. nigra* genomic DNA was labeled with Bio-11-dUTP (Fermentas) by nick translation and used as a probe. The blocks were generated by boiling *B. rapa* DNA for 30 min to produce DNA fragments of 300–500 bp. Anthers selected at meiosis were digested at 37 °C for approximately 70 min in an enzyme mixture containing 0.02% snailase (Beijing Baitai Biochem Co., China), 0.4% pectinase (Merck, Germany) and 0.6% cellulase "Onozuka" (Yakult Honsha Co., Ltd., Japan).

GISH was performed according to methods described by Cui et al. (2012). The hybridization signals of the *B. nigra* probe were discerned using Cy3-labeled streptavidin (Sigma Aldrich Co., Switzerland) and the chromosomes were counterstained with a 0.2% 4′-6-diamidino-2-phenylindole (DAPI) solution. The samples were mounted in Citifluor antifade medium. Photographs were taken using a computer-assisted fluorescence microscope (Nikon Eclipse 80i, Japan) with a Digital Sight camera.

AFLP marker analysis

AFLP marker analysis was performed based on methods described by Vos et al. (1995). The DNA was digested at 37 °C using 5 U EcoRI and 2 U MseI in a reaction volume of 25 μl, and the ends of the restriction digest fragments were ligated to the artificial adaptors (E-F: 5′-CTCGTAGACTGCGTACC-3′, E-R: 5′-AATTGGTACGCAGTC-3′ and M-F: 5′-GACGATGAGTCCTGAG-3′, M-R: 5′-TACTCAGGACTCATC-3′) (Sangon, China). The ligation product was amplified using pre-amplification primers (EA/MC), and the pre-amplified products were diluted (1:30) and then amplified using selective primers. Selectively amplified products were separated using electrophoresis on 6% denaturing polyacrylamide gels and silver stained. In this study, we selected 31 pairs of high-polymorphism AFLP primer EA/MC combinations for the whole-genome scan of the BC_1F_4 plants.

SSR marker analysis

The primer sequences for the SSR markers were obtained from the *Brassica* database (http://ukcrop.net). Initially, 220 primer pairs were randomly selected. Among these 220 primers, 85 SSR markers were assessed for successful PCR amplification and polymorphisms by testing the genomic DNA of the novel *B. napus* lines. PCR amplifications were performed in a volume of 10 μl containing 50 ng of genomic DNA, 10 × Taq buffer (containing Mg^{2+}), 10 mM dNTPs (Sangon, China), 0.2 U Taq DNA polymerase (Takara, Japan), and 5 μM forward and reverse primers (Sangon, China). The PCR reaction program was as follows: 94 °C for 2 min; 10 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s, with a decrease of 0.5 °C in the annealing temperature for each successive cycle; 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s; and a final extension at 72 °C for 5 min. The amplification products were separated using electrophoresis on 6% denaturing polyacrylamide gels, followed by silver staining. Finally, photographs were acquired to analyze the polymorphisms amplified by PCR.

Data statistics

The genetic similarity coefficient (GS) between accessions A and B was calculated by the formula $GS_{AB} = 2N_{AB}/(N_A + N_B)$, in which N_{AB} was the number of common bands shared by accessions A and B, and N_A and N_B were the total number of bands in accessions A and B, respectively. The genetic distance (GD) was calculated by the formula $GD_{AB} = -\ln(GS)$ (Nei and Li 1979). The data from the GS matrix among 67 genotypes were subjects to GD using the NTSYS-pc version 2.10e (Rohlf 1997). The introgression rate (IR) was calculated by the formula $IR_{AB} = N_A/(N_A + N_B)$. The correlation coefficient was calculated using Microsoft Excel.

Results

Morphology of hybrid progenies between *B. juncea* and *B. rapa*

There were significant variations in the morphology of the hybrid progenies between *B. juncea* and *B. rapa*.

The young plants of the hybrid progenies were divided into *B. rapa*-like, intermediate, *B. juncea*-like and other types based on characters, such as leaf shape, stem length, and florescence. Figure 2a, b shows the parents. Figure 2c shows the F_1 hybrid plant, *B. juncea*-like. Figure 2e–g shows the *B. rapa*-like, intermediate, and *B. juncea*-like BC_1F_3 plants, respectively.

The 48 F_1 plants between Luhuo and Menyuan were morphologically similar to *B. juncea* and male sterile. In total of 28 BC_1F_1 plants obtained from the F_1 plants pollinated by the *B. rapa* parent were investigated. Herein, *B. rapa*-like, intermediate and *B. juncea*-like plants accounted for 28.6, 46.4, and 25% of the BC_1F_1 population, respectively (Table 1). A total of 119 BC_1F_2 plants obtained from self-pollinating BC_1F_1 plants were investigated. In the BC_1F_2 population, the intermediate, *B. rapa*-like and *B. juncea*-like plants accounted for 54, 34.4 and 11.8%, respectively (Table 1). A total of 161 BC_1F_3 plants obtained from self-pollinating BC_1F_2 plants were investigated. Intermediate, *B. rapa*-like, *B. juncea*-like and additional plants accounted for 46.6, 32.3, 11.2 and 9.9% of the BC_1F_3 population, respectively (Table 1); the additional plants were different from the parent, intermediate, *B. rapa*-like and *B. juncea*-like plants. As shown in Table 1, the intermediate type comprised the majority of the BC_1F_1 plants; *B. rapa*-like and *B. juncea*-like plants were in the minority, with similar plant numbers. With an increase in self-pollination, the intermediate plants of the BC_1F_2 and BC_1F_3 populations accounted for approximately half, and the proportion of *B. rapa*-like plants was significantly higher than that of *B. juncea*-like plants. In addition, a similar phenotype was found between the novel BC_1F_4 *B. rapa* plants and the *B. rapa* parent

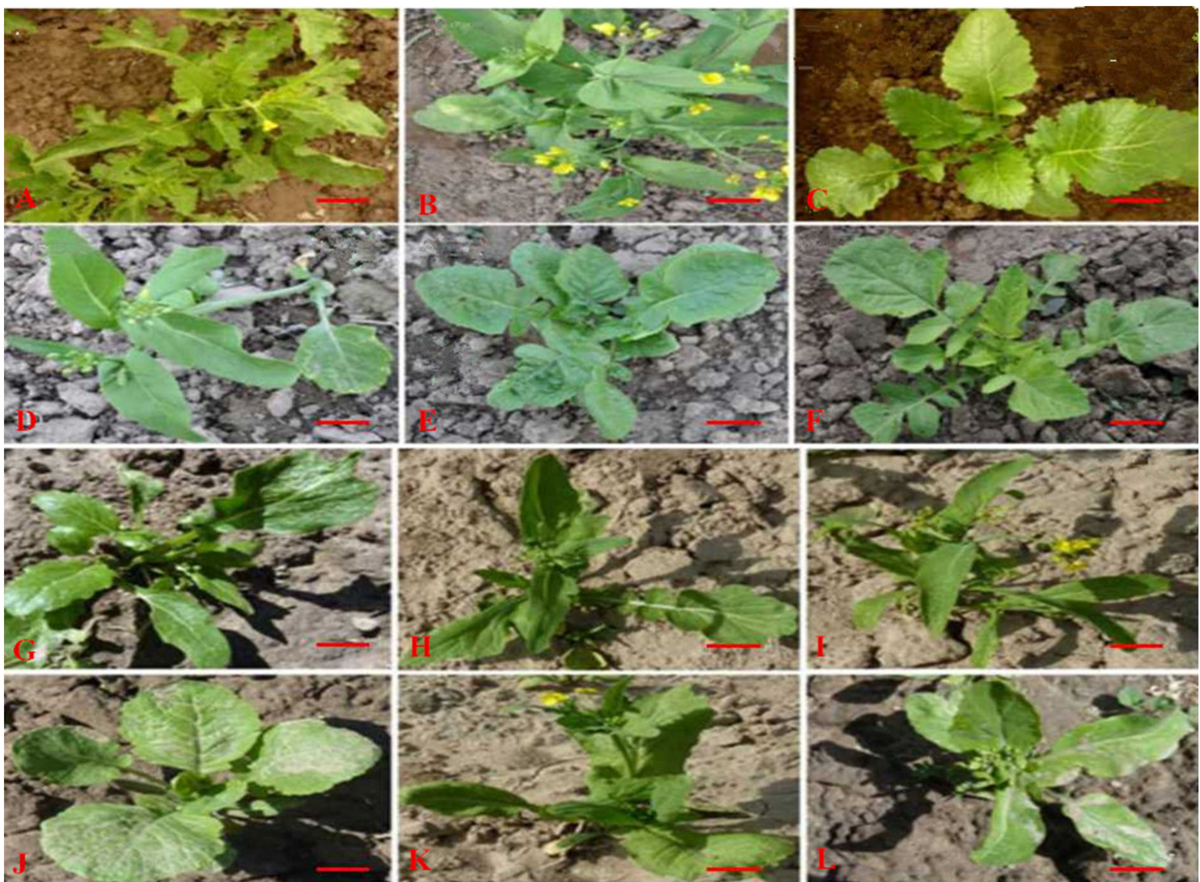

Fig. 2 Hybrid progenies between *B. juncea* and *B. rapa* and their parents **a** *B. juncea*-female parent. **b** *B. rapa*-male parent. **c** F_1 hybrid plant (*B,juncea*-like). **d** *B. rapa*-like (BC_1F_3). **e** Intermediate (BC_1F_3). **f** *B. juncea*-like (BC_1F_3). **g** N153-2 (BC_1F_4). **h** N151-3(BC_1F_4). **i** N26-1 (BC_1F_4). **j** N10-1 (BC_1F_4). **k** N169-1 (BC_1F_4). **l** N66-1 (BC_1F_4) (1 bar = 5 cm)

Table 1 Genetic variation in morphology among hybrid progenies between *B. juncea* and *B. rapa*

Type of plant	BC_1F_1		BC_1F_2		BC_1F_3	
	Number of plants	Percent of plants (%)	Number of plants	Percent of plants (%)	Number of plants	Percent of plants (%)
B. rapa-like	8	28.6	41	34.4	52	32.3
Intermediate	13	46.4	64	53.8	75	46.6
B. juncea-like	7	25	14	11.8	18	11.2
Other	0	0	0	0	16	9.9
Total	28	100	119	100	161	100

plants. The novel BC_1F_4 *B. rapa* plants included those with early flowering and yellow seed coat, but there were remarkable phenotypic differences among the individual BC_1F_4 plants (Fig. 2g–l).

Pollen viability observation of hybrid progenies between *B. juncea* and *B. rapa*

With in the increase of selfing times of the hybrid progenies from this interspecific cross, pollen viability gradually returned to normal (Table 2). The percent viable pollen of 28 BC_1F_1 plants was analyzed and found to average 34.8% and range from 0 to 84% among individual plants. The percent viable pollen of 81 BC_1F_2 plants was also analyzed and found to average 79.7% and range from 7 to 99%. In the BC_1F_3 plants, 158 individuals were analyzed; the percent viable pollen of 148 fertile individuals was > 93.7%, and the percent viable pollen of five individuals was < 30%. Therefore, the percent viable pollen of BC_1F_2 and BC_1F_3 plants was greater than that of BC_1F_1 plants.

GISH analysis of hybrid progenies between *B. juncea* and *B. rapa*

A total of 41 pollen mother cells (PMCs) were observed at diakinesis in the hybrid F_1 plants (Table S1), and eight B chromosomes were detected and labeled with the *B. nigra* probe. Of these 41 PMCs, 26.8% contained univalent B chromosomes; 65.9% of the PMCs contained partially homologous paired chromosomes in the B-genome that formed bivalents (Fig. 3a–c); and some PMCs contained A and B chromosomes that formed bivalents and multivalents. In total, 105 PMCs were observed at metakinesis I; these PMCs had lagged chromosomes that formed clavate bivalents to the majority of the A chromosomes and were tidily arranged in the equatorial plate. Both sides of the equatorial plate had lagged B chromosomes that existed in four to eight univalents; the other chromosomes were bivalents or multivalents (Fig. 3d). Furthermore, 157 PMCs (26.1%) had lagged chromosomes (Fig. 3f), and 14% of the PMCs had chromosome bridges. The A chromosomes had 10 II + 10 II, and the eight B chromosomes had 3 I + 5 I

Table 2 Genetic variation in pollen fertility among hybrid progenies between *B. juncea* and *B. rapa*

Percent viable pollen (%)	BC_1F_1		BC_1F_2		BC_1F_3	
	Number of plants	Percent of plants (%)	Number of plants	Percent of plants (%)	Number of plants	Percent of plants (%)
0–30	15	53.6				
30–50	4	14.3	3	3.7	1	0.6
50–80	8	28.6	22	27.2	4	2.5
> 80	1	3.5	53	65.4	148	93.7

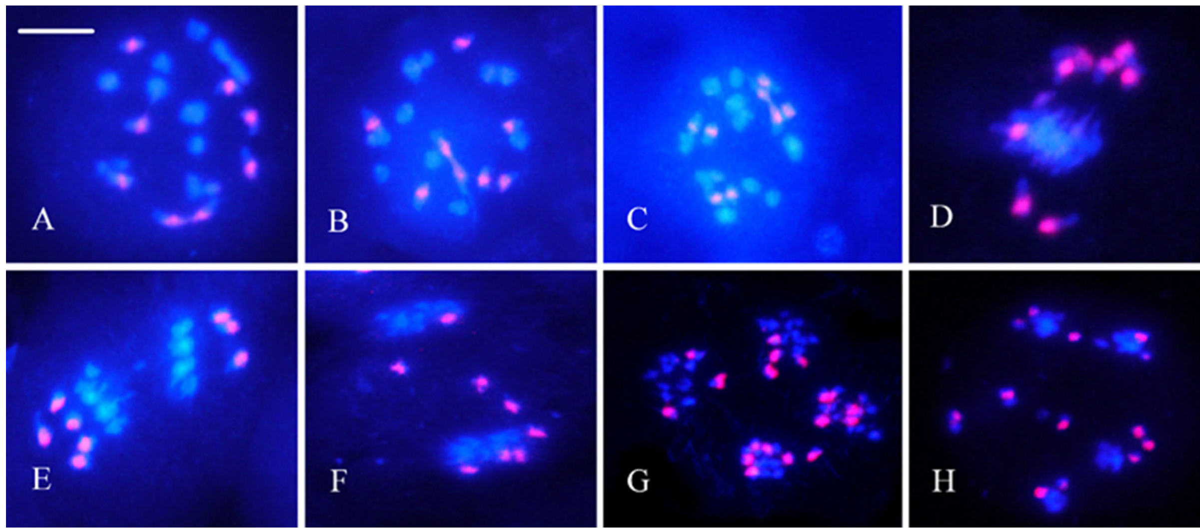

Fig. 3 GISH analysis of F_1. The blue color represents the DAPI staining of the chromosomes. The red color represents the labeling of the *B. nigra* probe. **a–c** Eight B-genome chromosomes were detected, which formed one, two and three bivalent chromosomes at diakinesis. **d** There were seven lagged B-genome chromosomes and one B-genome chromosome in the equatorial plate at metakinesis. **e** PMC divided into two poles and B-genome chromosomes separated as 3:5 at A I. **f** PMC divided into two poles and had three lagged and five normal segregated B-genome chromosomes that segregated as 1:4 at A I. **g** Lagged chromosomes, and B-genome chromosomes separated as 3:4:4:5 at A II. **h** Lagged B-genome chromosomes or fragments, and PMC divided into six parts (1 bar = 10 μm)

(Fig. 3e), 4 I + 4 I, and 2 I + 6 I that were observed at anaphase I (A I). 78 PMCs were detected at anaphase II (A II); the majority of PMCs containing B chromosomes divided into four daughter cells (Fig. 3g), 38.5% of PMCs had lagged chromosomes, and 28.2% of PMCs divided into three or five daughter cells (Fig. 3h).

A total of 13 BC_1F_1 plants were obtained from the F_1 × *B. rapa* cv. Mengyuan, and GISH was used to analyze (Table S1) and detect the number of B chromosomes introgression (Table 3). Nos. 140-1, 140-2, 141-2 and 143-2 were *B. rapa*-like and exhibited early flowering, a light-green leaf color, smooth leaves, and normal fertility. These plants had stable chromosome numbers (20 chromosomes), and four plants contained B chromosomes or fragments of the B-genome and were considered translocation lines of B chromosomes.

Nos. 140-1 and 140-2 had two B chromosomes, and Nos. 141-2 and 143-2 had three B chromosomes. No. 141-2 had 10 II + 10 II at A I, and the B chromosomes had 2 I + 1 I (Fig. 4a). Four daughter nuclei had ten chromosomes at A II, two daughter nuclei had one B chromosome, and the other nuclei had two B chromosomes (Fig. 4b). The meiosis the three B chromosomes in No. 141-2, along with their fertility, had almost recovered to normal. It was assumed that the pairing of the two B chromosomes and the partial homologous pairing between an additional B chromosome and A chromosome indicated that meiosis was normal.

Chromosomes of Nos. 144-1, 142-2 and 141-3 tended to intermediate phenotypes; the phenotypes and flowering phases were between those of the parents. The chromosome numbers were 22–25, with an additional 2-5 B chromosomes. There were 22 chromosomes of No. 144-1, which had two additional B chromosomes. Of 84 PMCs of No. 144-1, 83.3% exhibited 10 II + 2 I at diakinesis (Fig. 4c), and two B chromosomes existed as univalents, followed by 8 II + 2 III (3.6%) (Fig. 4d) and 11II (13.1%) (Fig. 4e) at diakinesis. A trivalent formed between two B chromosomes and one A chromosome, and 11 bivalents fromed between 20 A chromosomes and two B chromosomes, indicating homoeology between A and B chromosomes and between B and B chromosomes. There were 25 chromosomes of No. 141-3, with five additional B chromosome. A total of 41 PMCs exhibited two to five lagged chromosomes at metakinesis I (Fig. 4f); 46 PMCs (23.1%) exhibited lagged

Table 3 The number of additional B chromosomes in BC_1F_1

Plant number	Number of chromosomes	Number of B chromosomes	Plant type	Percent viable pollen (%)
140-1	20	2	*B. rapa*-like	61.2
140-2	20	2	*B. rapa*-like	43.3
141-2	20	3	*B. rapa*-like	79.3
143-2	20	3	*B. rapa*-like	50.6
144-1	22	2	Intermediate	55.4
142-2	24	4	Intermediate	22.9
141-3	25	5	Intermediate	84.4
143-4	26	6	*B. juncea*-like	15.4
142-3	26	6	*B. juncea*-like	0
366-5	26	6	*B. juncea*-like	22.3
140-5	26	6	*B. juncea*-like	61.1
142-5	26	6	*B. juncea*-like	14.4
140-4	44	16	*B. juncea*-like	63.7

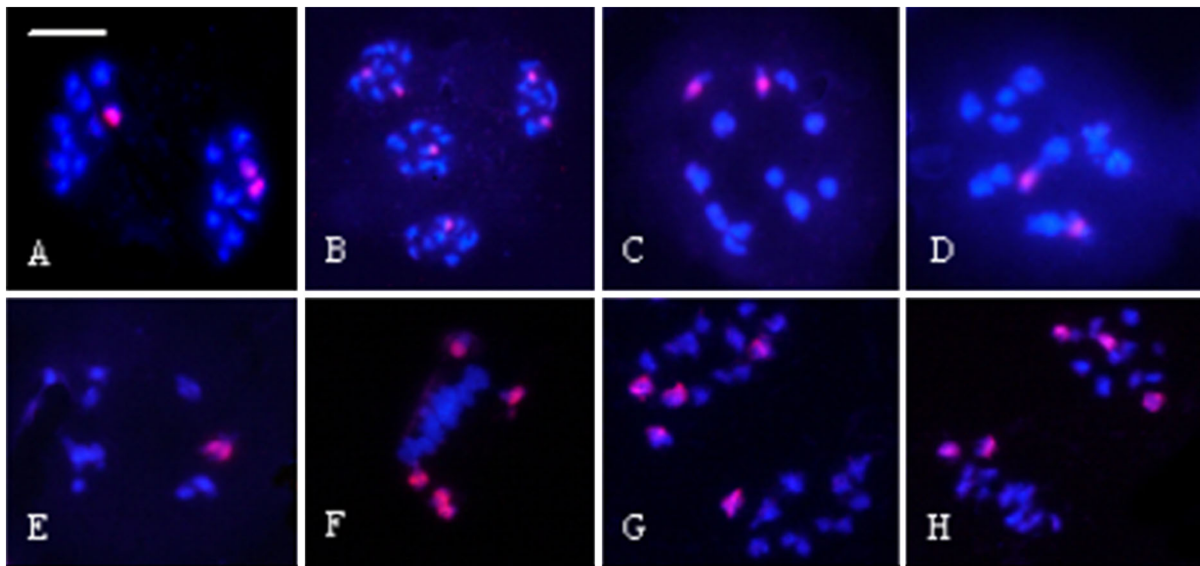

Fig. 4 GISH analysis of *B. rapa*-like and intermediate BC_1F_1. **a** No. 141-2 segregated as 10:10, which had three B-genome chromosomes that separated as 1:2 at A I. **b** Four daughter nuclei had ten chromosomes at A II. Two daughter nuclei had one B-genome chromosome, and the other nuclei had two B-genome chromosomes. **c** No. 144-1 had 10 II + 2 I at diakinesis, and two univalent chromosomes had two B-genome chromosomes. **d** No. 144-1 had 8 II + 2 III at diakinesis, and two trivalent chromosomes had two B-genome chromosomes. **e** No. 144-1 had 11 II at diakinesis, and one of the bivalent chromosomes had two B-genome chromosomes. **f** No. 141-3 had five lagged chromosomes at metakinesis I, which included five B-genome chromosomes. **g** No. 141-3 segregated as 11:15 and had six B-genome chromosomes that separated as 1:5 at A I. **h** No. 141-3 segregated as 12:13 and had five B-genome chromosomes that separated as 2:3 at A I (1 bar = 10 μm)

chromosomes at A I, followed by 11:14 (23.1%) (Fig. 4g) and 12:13 (27.0%) (Fig. 4h) segregations.

There were 26 chromosomes of Nos. 143-4, 142-3, 366-5, 140-5 and 142-5 which were *B. juncea*-like: tall and dark green, with rough and pinnatifid leaves and stocky stems. They exhibited late flowering, staminodes, small siliques and low seed set. A total of 18 PMCs (55.6%) had partial homologous pairing

between B and B chromosomes (Fig. 5a), 11.1% formed multivalent chromosomes between A and B chromosomes at diakinesis in chromosomes of No. 140-5, and the remaining (33.3%) retained univalent chromosomes. A total of 40 PMCs contained 2-6 lagged chromosomes, approximately 80.0% at metakinesis I (Fig. 5b). A total of 48 PMCs (81.2%) contained lagged chromosomes (Fig. 5c–e), and the chromosomes of 6.3% formed chromosome bridges at A I (Fig. 5f).

The phenotype of No. 140-4 included late-flowering; extended stigma; normal fertility; small siliques; low seed set; tall plant; stocky and large stem; pinnatifid basal; and lobed and striped leaves, making No. 140-4 *B. juncea-like* and mixoploid (2n = 20, 36, 41, 44, 54), with a preponderance of 2n = 44. The PMCs of No. 140-4 contained chromosomes of 2n = 44 (including 16 B chromosomes) and were *B. juncea*-like in morphology but had normal fertility. The percent viable pollen was approximately 63%, so we speculated that the 16 B chromosomes were an additional complete set of diploids and normal chromosomes paired in meiosis. Four PMCs contained 2-3 lagged chromosomes at metakinesis I. A total of 35 PMCs (8.6%) contained 2-6 lagged chromosomes, and the chromosomes of 5.7% formed chromosome bridges, followed by 10:6 (Fig. 5g), 9:7 (Fig. 5h) and 8:8 segregations of B chromosomes at A I. A total of 21 PMCs contained no lagged chromosomes or chromosome bridges, and the chromosomes of 9.5% contained abnormal tetrads (including trisections and five sections) at A II. The chromosomes of 31 PMCs (6.3%) had a micronucleus at the tetrad stage.

PMCs of the BC_1F_2 plants of three substitution lines and two AA-B addition lines were identified (Table S1). Nos. 814-1, 820-4 and 850-3 were substitution lines that had different B chromosome numbers. No. 814-1 had twenty chromosomes, including two B chromosomes (Fig. 6a). No. 820-4 had twenty chromosomes, including four B chromosomes, and pairwise coupling of B chromosome resulted in two bivalents at diakinesis (Fig. 6b); two B chromosome bivalents were tidily arranged at the equatorial plate at metakinesis (Fig. 6c), followed by 2:2 stable segregations of the four B chromosomes at A I (Fig. 6d). No. 830-5 had 20 chromosomes and contained two hybrid signals, one located at the end of a chromosome and the other located on a whole chromosome (Fig. 6f). The result indicated that No. 830-5 was a monosomic substitution to a translocation between an A and a B chromosome.

Nos. 830-4 and 892-5 were addition lines. No. 830-4 had 22 chromosomes, including two B chromosomes (Fig. 6e). No. 892-5 was mixoploid with 19, 21 and 22 chromosomes in PMCs and a preponderance of 21 chromosomes that accounted for approximately

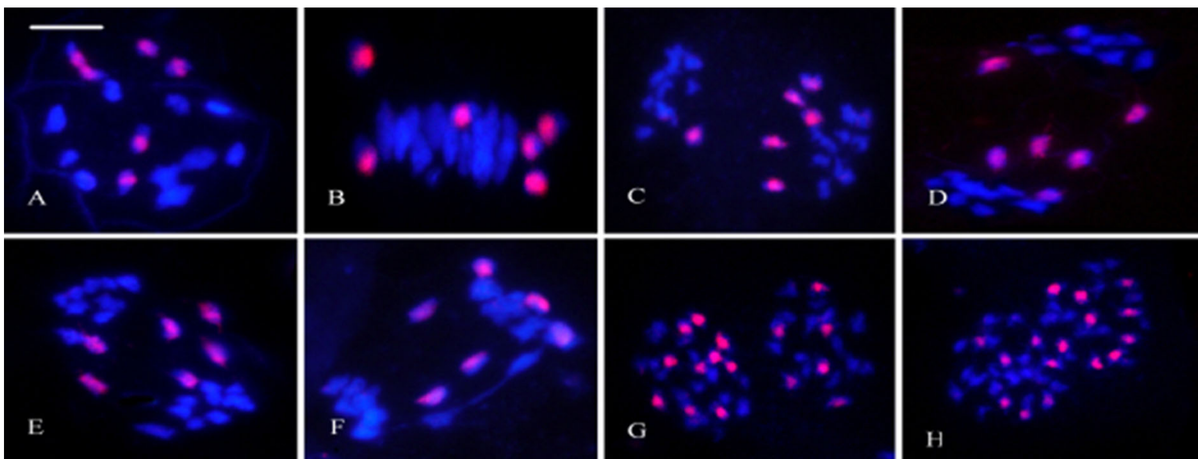

Fig. 5 GISH analysis of *B. juncea*-like BC_1F_1. **a** No. 140-5 had two B-genome chromosomes pairs that formed bivalent chromosomes at diakinesis. **b** No. 140-5 had lagged B-genome chromosomes at metakinesis I. **c** No. 140-5 had six lagged B-genome chromosomes that separated as 1:5 at A I. **d** No. 140-5 had six lagged B-genome chromosomes that separated as 2:4 at A I. **e** No. 140-5 had six lagged B-genome chromosomes that separated as 3:3 at A I. **f** No. 140-5 had lagged B-genome chromosomes that formed a chromosome bridge. **g** No. 140-4 had sixteen B-genome chromosomes that separated as 10:6 at A I. **h** No. 140-4 had sixteen B-genome chromosomes that separated as 9:7 at A I (1 bar = 10 μm)

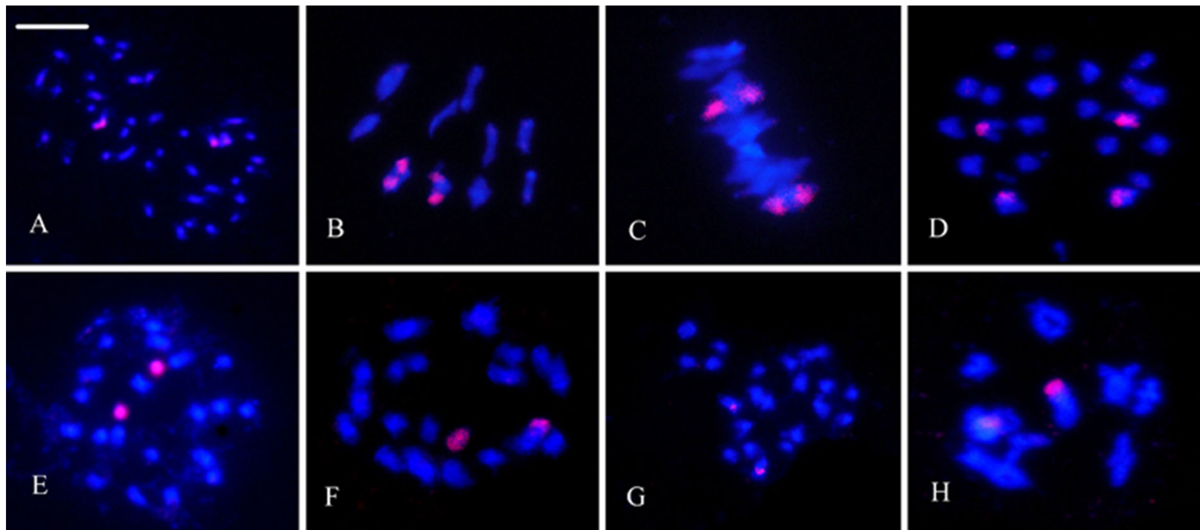

Fig. 6 GISH analysis of BC_1F_2. **a** No. 814-1 had as two B-genome chromosomes at A I. **b** No. 820-4 had as four B-genome chromosomes and two B chromosomes pairs that formed two bivalent chromosomes at diakinesis. **c** B-genome chromosomes of No. 820-4 paired as two bivalents and tidily arranged at the equatorial plate at metakinesis I. **d** Chromosomes of No. 820-4 separated as 10:10 and one signal in one polar group at A I. **e** No. 830-4 had twenty-two chromosomes, including two B-genome chromosomes in somatic cells. **f** No. 830-5 had twenty chromosomes. One B-genome chromosomes, signal was located at the end of a chromosome and the other was located on a whole chromosome. **g** No. 892-5 had twenty-one chromosomes. There were two B-genome chromosomes near the chromosome centromere. **h** No. 892-5 had as two B-genome chromosomes at diakinesis (1 bar = 10 μm)

45%. There were two hybrid signals in somatic cells (Fig. 6g) and located near the centromere during diakinesis of PMCs in meiosis phase (Fig. 6h). We speculated that a chromosome segment translocated while A and B chromosomes homoeologous pairing occurred near the centromere.

Observation of BC_1F_3 cytology and pollen staining

Through morphological investigation, cytological observation (Fig. 7a–e) and pollen staining (Fig. 7f) of the BC_1F_3 interspecific hybrid progenies between *B. juncea* and *B. rapa*, 124 novel *B. rapa* plants (AA, 2n = 20) were found to have obvious morphological differences, normal fertility and percent viable pollen ≥ 80% (Fig. 7f). According to cytological observation, PMCs had steady chromosome numbers of 10 II at diakinesis (Fig. 7b), had no lagged chromosomes at metaphase I (Fig. 7c), 10:10 segregation at A I (Fig. 7d), and 10 chromosomes of a tetrad nucleus at A II (Fig. 7e).

AFLP and SSR marker analysis of BC_1F_4 plants

A total of 60 BC_1F_4 plants were selected by AFLP and SSR analyses. AFLP analysis was conducted using 256 randomly selected primers. Among these markers, 31 pairs showed polymorphisms in two parents and check materials, and of 530 amplified bands, 519 (97.92%) were different. Each primer detected an average of 16.74 allele locations; the sizes of the bands were 80–1400 bp. There were 59 identical bands between *B. nigra* and *B.rapa* (Mengyuan) that had maximum GD; these lines each had 211 bands and 232 bands, respectively. *B. nigra* and *B. juncea* had the B-genome and produced B-specific bands. A total of 60 BC_1F_4 plants produced 13,054 bands, including 522 (4.4%) B-specific bands. This demonstrates that B chromosome(s) or chromosome segment(s) introgressed into hybrid progenies between *B. juncea* and *B. rapa* (Table 4).

SSR analysis was conducted using 220 primers designed from the A-genome (Fig. S1). Primer selection used two parents and two random progenies. A total of 85 primers had polymorphic bands and amplified 265 bands, 258 (97.4%) of which were

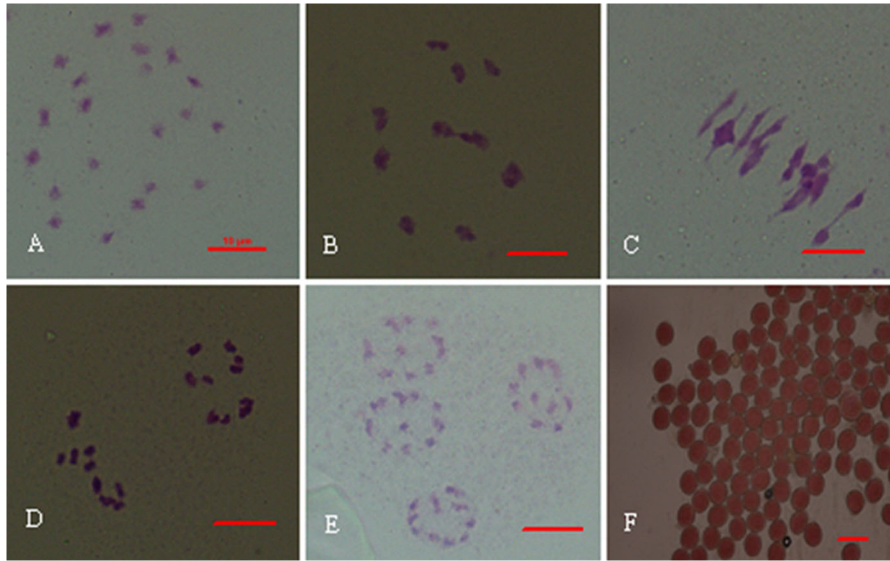

Fig. 7 Cytological observation of novel BC_1F_3 *B. rapa.* **a** Chromosome numbers of somatic cells (2n = 20). **b** Chromosomes separated as 10 II in diakinesis. **c** No lagged chromosomes in metaphase I. **d** Chromosomes separated as 10:10 in A I. **e** A tetrad nucleus (n = 10) in A I. **f** Pollen staining (1 bar = 10 μm)

different. Each primer detected an average of 3.04 allele locations, and the band sizes were 65–430 bp. *B. juncea* and *B. rapa* contained the A-genome, which underwent changes to long-term evolution. The A-genome of *B. juncea* indicated as A^J. The A-genome (A1-A10) of *B. juncea* was introgressed into the hybrid progenies, and the IR ranged from 1.0 to 42.7%. The A6 IR was the highest, whereas the A10 IR was the lowest (Table 5).

The A^J and B chromosome(s) or chromosome segment(s) of *B. juncea* introgressed into the 60 novel *B. rapa* (Fig. S2). The average IR of A^J was approximately 20.7%, and ranged between 12.7 and 29.2%. The IR of No. 28 was the highest, whereas the IR of No. 54 was the lowest (Table 4). The average IR of the B-genome was approximately 3.7% and ranged between 1.5 and 8.1%. The IR of Nos. 17 and 38 was the highest, whereas the IR of Nos. 19 and 20 was the lowest (Table 4). In the 60 novel *B. rapa* plants, the A-genome IR was significantly higher than that of the B-genome. The GD between Mengyuan and the novel *B. rapa* was 0.224–0.538. The GD of No. 16 was the highest, whereas the GD of No. 56 was the lowest. The correlation coefficients were significantly different among the GD between the novel *B.rapa* and *B.rapa* versus Menyuan and among the IR of the A and B chromosome(s) or chromosome segment(s) of *B. juncea* introgressed to the novel *B. rapa*, which was 0.52 and 0.83, respectively. The results showed that the more A^J and B chromosome(s) or chromosome segment(s) of *B. juncea* that were introgressed into the novel *B. rapa*, the greater the GD between the novel *B. rapa* and the parent vs. *B. rapa* was.

Development of novel *B. rapa*

The seed color of *B. juncea* cv. Luhuo was yellowish-brown, that of *B. rapa* cv. Mengyuan was red-brown, and that of the F_1 plants was brown. Five shallow yellow-seed individuals were discovered in BC_1F_2; three individuals maintained this seed color, but that of the others segregated into brown and yellow in progenies. Five yellow-seed individual BC_1F_2 plants were self-pollinated to obtain 57 BC_1F_3 individuals that showed different degrees of seed color (Fig. 8). We also investigated the somatic chromosome numbers of 28 yellow-seed BC_1F_3 individuals that were 20. Thus, most of the yellow-seed BC_1F_3 individuals were *B. rapa*-type (Table S2).

Some of the BC_1F_4 plants flowered early, a phenotype similar to that of the *B. rapa* parent, but there were remarkable phenotypic differences among the various plants. N153-2 was less branched, with elongated leaves and early flowering (Fig. 1g). N151-

Table 4 Correlations between genetic distance and introgression rate of BC_1F_4

Number of plants	Introgress- ion rate of the A^J-genome	Introgress-ion rate of the B-genome	Genetic distance (cM)	Number of plants	Introgress- ion rate of the A^J-genome	Introgress-ion rate of the B-genome	Genetic distance (cM)	Number of plants	Introgress- ion rate of the A^J-genome	Introgress-ion rate of the B-genome	Genetic distance (cM)
2	23.1	3.8	0.283	22	28.2	3.8	0.312	42	20	2.7	0.256
3	19.8	3.8	0.245	23	18.3	4.6	0.279	43	20.7	3.3	0.261
4	20.7	3.4	0.256	24	29.2	3.3	0.303	44	24.6	6.9	0.281
5	16.1	3.4	0.241	25	27.6	5	0.369	45	14.2	2.9	0.241
6	19.2	3.4	0.263	26	27.2	4.1	0.313	46	17.7	3.2	0.266
7	22.2	1.9	0.251	27	22.7	3.4	0.269	47	21.7	3.5	0.283
8	21	3	0.261	28	20.6	4.9	0.274	48	22.2	4.5	0.246
9	16.9	2.3	0.248	29	21.6	1.8	0.251	49	18.9	3.8	0.259
10	24.8	3.3	0.294	30	18.5	1.8	0.235	50	12.7	3.9	0.248
11	23.1	2.4	0.261	31	19	3.3	0.256	51	14.5	4.8	0.256
12	24	3.3	0.315	32	14.8	3.8	0.271	52	19.2	4.7	0.246
13	21.6	8.1	0.538	33	13.9	2.4	0.233	53	19.4	3.5	0.224
14	23.5	3.2	0.268	34	20.5	8.1	0.291	54	24.5	4	0.271
15	21.1	1.5	0.263	35	26	5.6	0.286	55	21.8	3.9	0.276
16	22.4	1.5	0.279	36	18	5.3	0.402	56	21.3	4.6	0.293
17	25	3.6	0.286	37	19.8	2.3	0.259	57	15.5	2.8	0.23
18	21.9	4.1	0.258	38	18.9	3.4	0.246	58	27.7	5.5	0.261
19	22	4.8	0.271	39	15.4	4.1	0.24	59	19.3	4.7	0.251
20	17.8	1.6	0.243	40	17.9	3.7	0.248	60	19	3.6	0.274

Coefficient of correlation: 0.52** 0.83**

**Represent significant differences at $P < 0.01$

0.52 and 0.83 indicate significant correlation of novel *B. rapa* with *B. rapa* versus Menyuan and *B. juncea*, respectively

Introgression rate $(\%) = \left(A^J - \text{specific alleles or } B - \text{specific alleles/total alleles}\right) \times 100\%$

Table 5 Alleles of the A-genome of *B. juncea* introgress into novel *B. rapa*

Chromosomes	Number of primers	A^J-specific alleles	Total alleles	Introgression rate (%)
A1	11	149	805	18.5
A2	7	59	546	10.8
A3	8	212	625	33.9
A4	7	95	751	12.6
A5	8	109	600	18.2
A6	7	202	473	42.7
A7	6	261	623	41.9
A8	8	243	772	31.5
A9	16	223	1539	14.5
A10	7	6	599	1

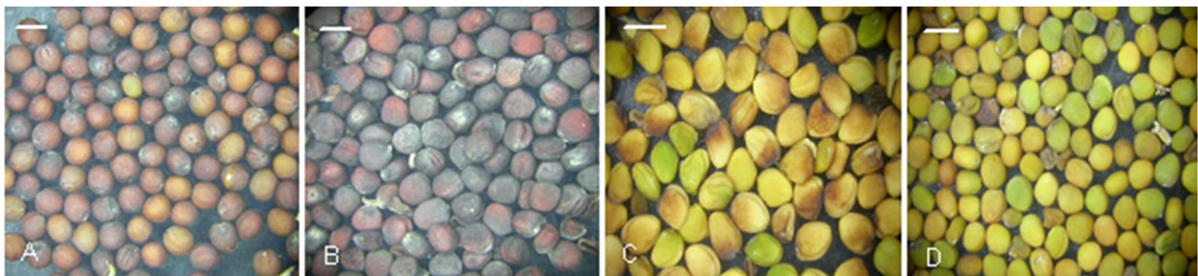

Fig. 8 Seed color of the parents and hybrid progenies between *B. juncea* and *B. rapa.* **a** *B. juncea*. b *B. rapa*. **c** 897-3 (BC_1F_3 plant). **d** 874-5 (BC_1F_3 plant) (1 bar = 3 mm)

3 exhibited a pinnatipartite leaf of the basal stem, symmetrical and orderly leaves on the side lobes, and an irregular oval leaf at the top of the stem (Fig. 1h). N26-1 had dark-green leaves, long petioles, and long, oval leaves (Fig. 1i). N10-1 was compact, with waxy and long, oval leaves (Fig. 1j). N169-1 displayed early flowering, wide and compact leaves and less branching (Fig. 1k). N66-1 had circular, waxy, wide and compact leaves and long petioles (Fig. 1l). Other traits, such as yield and oil content, were not assessed. These results showed that the novel *B. rapa* plants obtained by interspecific hybridization between *B. juncea* and *B. rapa* varied widely, broadening genetic diversity and enriching germplasm resources.

Discussion

B. juncea has many valuable agronomic characteristics, many of which have been transmitted into *B. rapa,* which can broaden its genetic diversity. In this study, the studied seeds included those resulting from a cross between *B. juncea* cv. Luhuo as the female parent and *B. rapa* cv. Mengyuan as the male parent, as well as some novel *B. rapa* with valuable traits, i.e., early flowering and yellow-seed. Unfortunately, seeds were not obtained using *B. rapa* as the female parent and *B. juncea* as the male parent. Similar studies have demonstrated that progeny from such crosses are rare. The reason for this result may be lower pairing between the B-genome and A- or C-genome, which are distantly related (Attia et al. 1986; Mason et al. 2010; Tan et al. 2017), and difficultly obtaining seeds. However, there is little evolutionary difference between the A-genome and the C-genome (Attia et al. 1987), as mentioned in previous reports in *Brassica* species (Leflon et al. 2006; Wen et al. 2008; Bennett et al. 2012; Li et al. 2013; Rahman et al. 2015).

With increased backcrossing, plants with viable pollen continued to be produced. Consistently, the percent viable pollen (0 $\sim$ 30%) of BC_1F_3 plants was 3.2%, much lower than that of BC_1F_1 plants (53.6%), but the percent viable pollen (> 80%) of BC_1F_3 plants was 93.7%, much higher than that of BC_1F_1 plants (3.5%) (Table 2). The reason for this result might be

the normal pairing of more homologous and homologous chromosomes through increased backcrossing. Pollen stainability observed in *B. juncea* × toria and *B. juncea* × yellow sarson hybrids was, on average, 16.5 and 20.4% in F_1 plants and 39.9 and 43.2% in F_2 plants, respectively (Choudhary et al. 2002). Pollen fertility increased with cross generation.

The *B.juncea*-like morphology of the *B. juncea* × *B. rapa* F_1 hybrids in this study contrast with the results of Choudhary et al. (2002) and Röbbelen (1960), who found an intermediate morphology. The majority of the 28 chromosomes were 10 II + 8 I, which was observed in the PMCs with univalent, bivalent and trivalent chromosome pairing. Such variation could accounted for the differences in the genotypes of the species involved in the production of the hybrids.

The occurrence of 10 II + 8 I in the majority of the PMCs of the F_1 hybrids (2n = 28) could be attributed to the homologous pairing of 10 chromosomes belonging to the A-genome, one each derived from *B. juncea* (AABB, 2n = 36) and *B. rapa* (AA, 2n = 20), leaving eight chromosomes of the B-genome as V + IV+ III + II + I. The occurrence of three additional lines and seven translocation lines might be due to autosyndesis between A- and B-genome chromosomes. This would result in structural similarities in a few chromosomes of the A- and B-genome. The occurrence of multivalents in the form of trivalents, quadrivalents and pentavalents indicated the presence of duplicate segments on the chromosomes or heterozygous translocation (Röbbelen 1960). The presence of the A- and B-genomes suggested the possibility of multivalent associations in hybrid progenies. Such affinity revealed allosyndetic pairing and provided the basis for genetic exchange between the genomes. Maternal- and intermediate-type plants were frequent among the hybrid progenies. Wide phenotypic variation and transgressive segregation for many traits in the hybrid progenies might have resulted from recombination and/or eventual segregation of aneuploid forms arising in the populations.

Introgressive hybridization, the incorporation of genetic materials from one species into another by wide hybridization and repeated backcrossing, plays a vital role in the evolution of plant species (Guttman 2001), genetic modification and enriching the gene pool for breeding (Anamthawat-Jonsson 2001), as evidenced in abundance (Li et al. 1995, 2005, 2006; Hua et al. 2006; Chen et al. 2007; Mason et al. 2010; Tan et al. 2017). AFLP and SSR results showed that A-genome transmission was higher than that of the B-genome. The reason for this result is likely that pairing and recombination preferentially occurred between homologous chromosomes in triploid AAB hybrids in F_1 plants as well as between homologous chromosomes at a low rate (Tan et al. 2017). This study also suggested that B-genome chromosomes can be introgressed into hybrid progenies (BC_1F_1, BC_1F_2, BC_1F_3 and BC_1F_4).

Addition lines and translocation lines could be used to increase the genetic diversity of *B. rapa* and provide new breeding materials for genetic improvement in Qinghai-Tibet. They could also be used to study the origin and evolution of the A- and B-genomes. In this study, we report the successful development of novel *B. rapa* lines with early flowering and yellow-seed phenotypes and many unidentified characters (i.e., resistance, quality, and yield). These lines serve as new genetic sources for *B. rapa* breeding through successive selections of the progenies from one interspecies hybrid between *B. rapa* and *B. juncea*.

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Authors' contribution ZZ and DD designed and managed this study. CT and YN performed the experiments and analyzed the data. CT wrote the manuscript. QY designed and executed the artificial synthesis of *B. rapa*.

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Compliance with ethical standards

Conflict of interest We declare that we have no financial or personal relationships with other people or organizations that can inappropriately influence our work. There are no professional or other personal interests of any nature in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "Production and genetic analyses of novel *Brassica rapa* L. introgressions from interspecific crosses with *Brassica juncea* L. landraces native to the Qinghai-Tibet Plateau".

Ethical approval This article does not describe any studies involving human participants or animals.

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