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ORIGINAL PAPER

A large-scale introgression of genomic components of *Brassica rapa* into *B. napus* by the bridge of hexaploid derived from hybridization between *B. napus* and *B. oleracea*

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Abstract *Brassica rapa* (AA) has been used to widen the genetic basis of *B. napus* (AACC), which is a new but important oilseed crop worldwide. In the present study, we have proposed a strategy to develop new type *B. napus* carrying genomic components of *B. rapa* by crossing *B. rapa* with hexaploid (AACCCC) derived from *B. napus* and *B. oleracea* (CC). The hexaploid exhibited large flowers and high frequency of normal chromosome segregation, resulting in good seed set (average of 4.48 and 12.53 seeds per pod by self and open pollination, respectively) and high pollen fertility (average of 87.05 %). It was easy to develop new type *B. napus* by crossing the hexaploid with 142 lines of *B. rapa* from three ecotype groups, with the average crossability of 9.24 seeds per pod. The genetic variation of new type *B. napus* was diverse from that of current *B. napus*, especially in the A subgenome, revealed by genome-specific simple sequence repeat markers. Our data suggest that the strategy proposed here is a large-scale and highly efficient method to introgress genomic components of *B. rapa* into *B. napus*.

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Introduction

Brassica napus (rapeseed, AACC, $2n = 38$) originated from a spontaneous hybridization between *B. rapa* (AA, $2n = 20$) and *B. oleracea* (CC, $2n = 18$) (U N 1935). Although it was domesticated as a cultivated crop only 400 years ago (Federico and Maria 2011; Gómez-Campo 1999), it has become the second most important oilseed crop after soybean in the world. As compared to *B. rapa*, *B. napus* possesses advantages in seed yield and disease resistance (Chen 2010; Liu 2000), and as a result *B. napus* has been replacing *B. rapa* as the major oilseed crop in some regions, such as China where *B. rapa* was a traditional and predominant oilseed crop before the 1960s (Liu 2000).

However, the narrow genetic basis has limited the improvement of current *B. napus* partly due to its intensive modern breeding and short history of origination and domestication (Becker et al. 1995; Girke et al. 2012; Seyis et al. 2003). For example, the modern breeding objective in seed quality (double-low, low erucic acid and low glucosinolate) has increases the chance of erosion of the genetic basis of rapeseed. Further, current *B. napus* has been derived from spontaneous hybridizations between a limited number of parental species genotypes, resulting in limited variation from parental species (Allender and King 2010; Mei et al. 2011a).

On the other hand, *B. rapa* with more than 6,000 years of domestication history widely distributes in the world and has enormous variability in morphology, agronomic characteristic and DNA structure (Gómez-Campo 1999; Zhao et al. 2005). Hence, it is an important breeding program to improve *B. napus* by use of *B. rapa* (Liu 2000; Mei et al. 2011b; Qian et al. 2006). In this study, we proposed a strategy for improving the current *B. napus* by crossing the

hexaploid (AACCCC) derived from *B. napus* and *B. oleracea* with *B. rapa*. Our data suggest relative stability of the hexaploid, high crossability between the hexaploid and *B. rapa* and diverse genetic variation of the new type *B. napus* from current *B. napus*, indicating that the strategy proposed here is a large-scale, highly efficient method to introgress genomic components of *B. rapa* into *B. napus*.

Materials and methods

Plant materials

To develop new type *B. napus*, a panel of *B. rapa* lines with wide genetic variation (Supplementary Material S1), including 9 spring, 6 winter and 127 semi-winter lines from Europe, Canada and China were employed to cross with a hexaploid derived from an interspecific hybridization between an elite cultivar of *B. napus* ('Zhongshuang 9' with double-low seed quality, 35.68 μmol/g glucosinolate and 0.55 % erucic acid) and a line of *B. oleracea* var. *acephala* ('SWU 01' with double-high seed quality, 155.23 μmol/g glucosinolate and 38.57 % erucic acid) via embryo rescue according to the method of Wen et al. (2008) (Fig. 1). The hybrid individuals between the hexaploid and *B. rapa* with more than 90 % of pollen fertility and 15 seeds per pod were chosen and referred to as new type *B. napus*.

To evaluate the genetic diversity between the new type and current *B. napus*, a set of current *B. napus* was randomly selected from three diverse gene pools (Becker et al. 1995; Bus et al. 2011; Diers and Osborn 1994; Hasan et al. 2006; Qian et al. 2006), composed of 15 spring, 16 winter and 14 semi-winter accessions and compared with 76 new type *B. napus* lines derived from 7 spring, 6 winter and 45 semi-winter *B. rapa*, including 58 F_1 lines and 18 F_2 lines (Supplementary Material S1). The seed quality of hexaploid progenies was assayed using near-infrared reflectance spectroscopy.

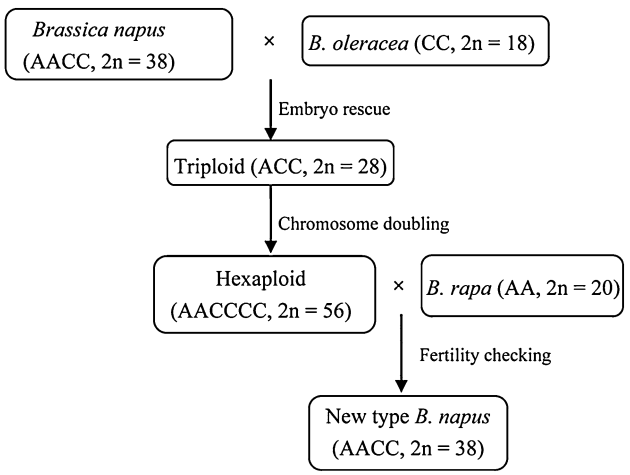

Fig. 1 Breeding diagram for developing new type *B. napus* carrying the genomic components of *B. rapa*

Cytological analysis

The ovaries from young buds were collected and treated with 8-hydroxyquinoline for 3–4 h at room temperature, fixed in Carnoy's solution (V_{ethanol}:$V_{\text{acetic acid}}$ = 3:1) and stored at 4 °C for chromosome number counting. The young buds were collected and fixed directly in Carnoy's solution and stored at 4 °C for meiosis study. To observe germination of *B. rapa* pollen and growth of pollen tube on the stigma of the hexaploid, the pistils were collected 10 h after pollination and fixed in Carnoy's solution for 24 h, treated with 8 mol/l NaOH for 8 h, stained with 0.1 % aniline blue solution and examined under the fluorescence microscope at room temperature (Yao et al. 2004).

The pollen grains from three flowers of each plant tested were stained with 1 % acetocarmine; more than 300 pollen grains were observed under the microscope. The percentage of stainable pollen grains was calculated to measure pollen fertility.

To investigate the chromosome behavior of the hexaploid (AACCCC), fluorescent in situ hybridization (FISH) was performed according to the protocol of Leitch and Heslop-Harrison (1994) with some modifications. The probe was developed by extracting DNA of the C-genome-specific BAC clone, BoB014O06, which can hybridize with the C chromosomes, but not with the A chromosomes in *Brassica* (Howell et al. 2002), and labeling with Bio-11-dUTP by random priming. Genomic DNA of *B. rapa* was sheared in boiling water for 15 min and was used as a block. An enzyme mixture containing 0.6 % cellulose "Onozuka" (Yakult Honsha, Japan), 0.4 % pectinase (Merck, Germany) and 0.5 % Snailase (Sabc, China) was used to decompose the cell wall of pollen mother cells at 37 °C for 95 min. The chromosomal DNA on each slide was hybridized with 100 ng probes, 1,000 ng genomic DNA of *B. rapa* and 100 ng ssDNA overnight in a mixture solution, containing 50 % deionized formamide, 2 × SSC, 10 % dextran sulfate and 0.5 % SDS. The hybridization signals of the BAC clone probe were detected using Cy3-labeled streptavidin (Sigma, USA), and chromosomes were counterstained with 0.2 % 4′-6-diamidino-2-phenylindole (DAPI) solution (Roche, BaseI, Switzerland), mounted in antifade solution (Vector) and examined under a fluorescent microscope (Nikon Eclipse 80i, Japan) equipped with a CCD camera. Images were processed using the software of Adobe Photoshop version 8.0.

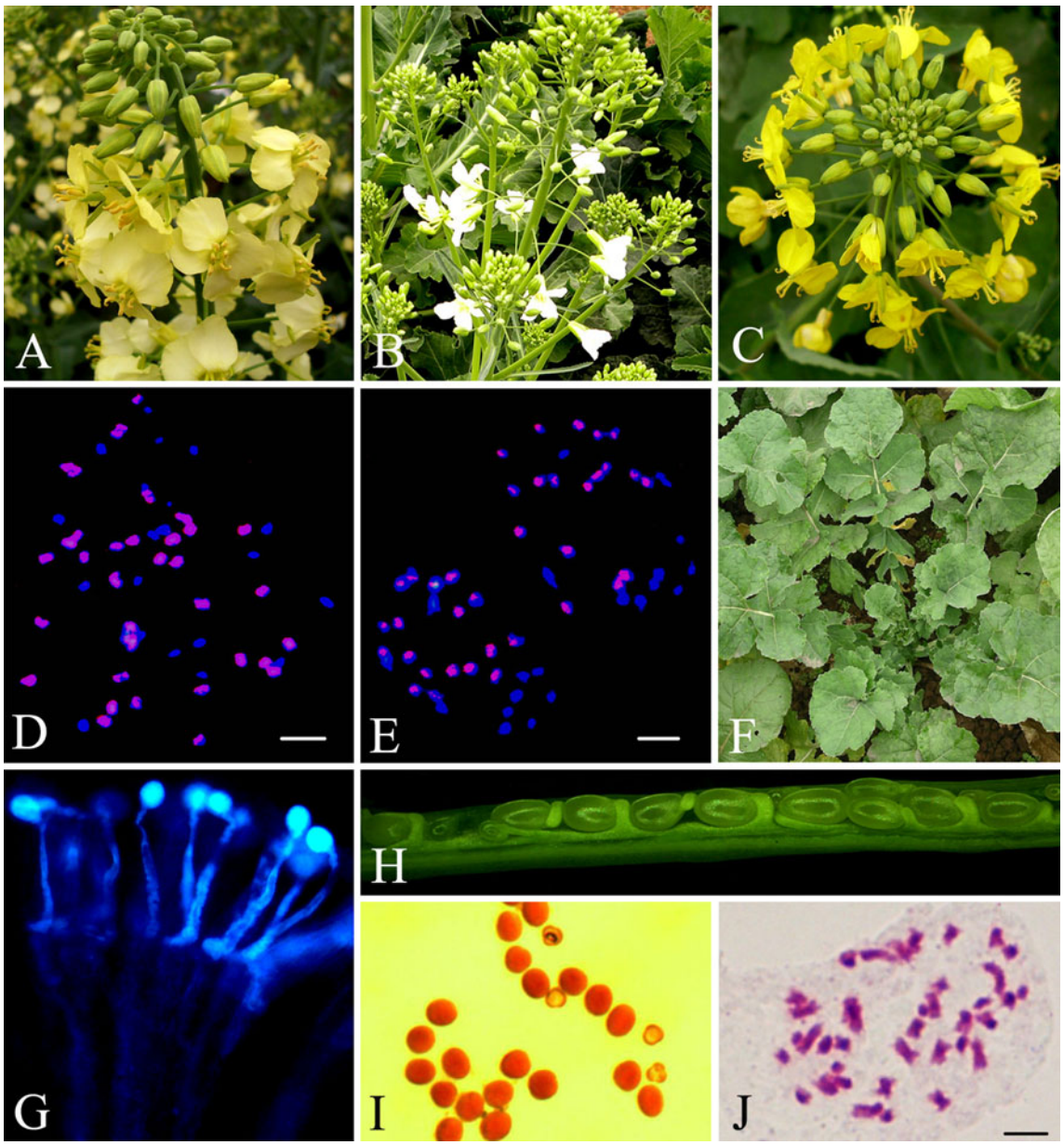

Fig. 2 Morphological and cytological characterizations of the hexaploid and new type *B. napus*. Inflorescences of hexaploid S0 (**a**) derived from *B. oleracea* (**b**) and *B. napus* (**c**). **d** One ovary cell of hexaploid S0 plant with chromosome pattern of 36C + 20A. Chromosomes were counterstained with 4′-6-diamidino-2-phenylindole solution (*blue*), while the C-subgenome chromosomes were labeled *red* by the probe BoB014O06. **e** One PMC of hexaploid S0 at AI with chromosome segregation of (18C + 10A):(18C + 10A). **f** One plant of new type *B. napus* at seedling. **g** Pollen grains of *B. rapa* grown well on the stigma of the hexaploid 10 h after pollination. **h** Embryos in the pod of the hexaploid developed well 15 days after pollination by *B. rapa*. **i** Pollen fertility in a new type *B. napus*. **j** One ovary cell of new type *B. napus* with $2n = 38$. *Bar* 5 μm (color figure online)

Genetic diversity evaluation

The genomic DNA was isolated from young leaves using the CTAB method. Fingerprints of genotypes were developed with 153 primer combinations of simple sequence repeats (SSR) (Supplementary Material S2). According to the information from multiple segregating populations (Mei et al. 2013; Piquemal et al. 2005; Shi et al. 2009), 69 of the 153 primer combinations amplified specific polymorphic loci in the A subgenome of *B. napus*, 81 in the C subgenome and 3 were unclear. The SSR bands were described by absence (0) or presence (1).

The genetic distance (GD) between accessions X and Y was calculated using the formula, $GD_{xy} = 1 - N_{xy}/(N_x + N_y)$, where N_{xy} is the number of common bands shared by accession X and Y, and N_x and N_y are the total

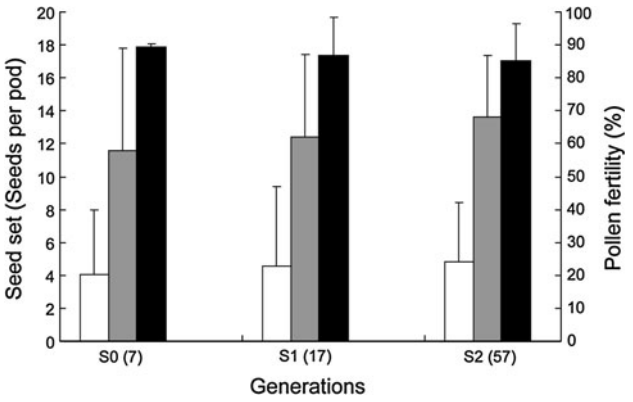

Fig. 3 Seed set by self-pollination (*white*) and open pollination (*gray*) along with pollen fertility (*black*) in the hexaploid *S0* (7 individuals), *S1* (17 individuals) and *S2* (57 individuals). The mean values (*columns*) and a half of the standard deviation (*bars*) are given

number of bands in accession *X* and *Y*, respectively (Nei and Li 1979). The data from the GD matrix among 121 genotypes were subjected to principal component analysis (PCA) using the NTSYS-pc version 2.1 (Rohlf 1997).

The gene diversity was calculated with the formula of Nei (1973), where $H = 1 - \sum X_i^2$, where X_i is the frequency of the *i*th allele.

Results

Development of new type *B. napus*

One embryo of interspecific hybrid between *B. napus* (Zhongshuang 9) and *B. oleracea* var. *acephala* (SWU 01) was rescued and cloned via asexual reproduction. A total of 184 clones were developed and transplanted in the field. They exhibited intermediate phenotypes between the two parents (Fig. 2a–c). Seven of the 184 clones with 1.5-fold larger flowers than others were identified to have $2n = 56$ chromosomes, 20 from A subgenome and 36 from C subgenome as revealed by FISH (Fig. 2d). Therefore, these plants with the karyotype of AACCCC might have been derived from spontaneous chromosome doubling during subculturing of the hybrid triploid (ACC). The individuals with large flowers were selected from two successive selfing progenies of the hexaploid (S1 and S2) for subsequent research.

No significant differences were found for seed set and pollen fertility among the three successive generations of the hexaploid (S0, S1 and S2). Along with the increase of the generations, the seed set of hexaploid was improved a little (Fig. 3). The mean value of the three generations of hexaploid was 87.05 % for pollen fertility, with 4.48 and 12.53 seeds per pod by self and open pollination, respectively (Fig. 3). The fertility of the hexaploid was much better than

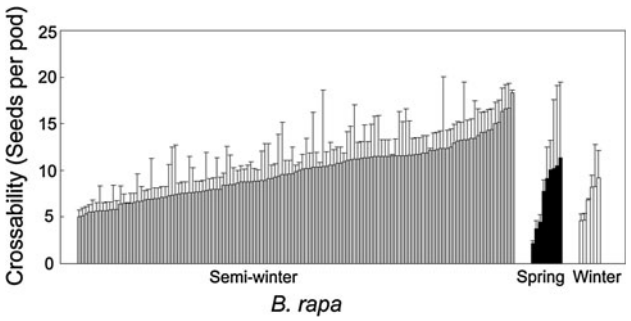

Fig. 4 Crossability of the hexaploid with 142 *B. rapa* accessions from three ecotypes. The mean values (*columns*) and a half of the standard deviation (*bars*) are given

that of the triploid which had 8.7 % of pollen fertility, nearly no seed after self-pollination and a few seeds after open pollination, lower than parental *B. napus* which possessed 96.36 % of pollen fertility, 19.68 and 20.16 seeds per pod by self and open pollination, respectively.

The finding of relatively good fertility in the hexaploid was in accordance with the observation of its chromosome behavior at meiosis. Among 126 PMCs of hexaploid S0, 88 (69.84 %) PMCs exhibited disomic inheritance with the pattern of chromosome distribution (10A + 18C) : (10A + 18C) at anaphase I detected by FISH (Fig. 2e), followed by (10A + 16C) : (10A + 20C) (21.43 %) and (10A + 17C) : (10A + 19C) (4.76 %), resulting in a relatively high frequency of euploid progenies. The chromosomal configuration at diakinesis of the hexaploid S0 was 0.087I + 15.308II + 0.048III + 6.212IV + 0.048VI + 0.001VIII.

Subsequently, 76 hexaploid individuals, including 17 S1 and 59 S2 with large flowers were selected randomly and their chromosome numbers were detected. The result showed that 74 individuals (97.37 %) had the same chromosome number as the hexaploid S0 ($2n = 56$). However, wide phenotype variations were detected in the progenies of hexaploid. For example, the interval of flowering time between early and late individuals was more than 1 month, and the content of glucosinolate in seed ranged from 35.61 to 108.66 μmol/g among 84 individuals tested in the hexaploid S2.

To investigate the crossability between the hexaploid and *B. rapa*, 142 accessions of *B. rapa* from three ecotypes were used as male parents to cross with 52 individuals of the hexaploid S1. The pollen tube of *B. rapa* could normally grow on the stigma of the hexaploid and the embryos of hybrids developed well when randomly testing nine hybrids derived from three ecotypes of *B. rapa* (Fig. 2g–h). No significant differences for the crossability of the hexaploid with the three ecotypes of *B. rapa* were found ($P = 0.90$). The average was 9.53, 8.18 and 10.01 seeds per pod for the crossability of the hexaploid with spring,

Table 1 Average genetic distance and gene diversity (mean ± SD) within and between new type *B. napus* (N group, 76 lines) and current *B. napus* (C group, 45 lines) revealed by 153 SSR primer combinations, including 69 A and 81 C subgenome-specific primer combinations

Group	Genetic distance			Gene diversity
	A subgenome	C subgenome	All	
N group	0.35 ± 0.12	0.30 ± 0.12	0.32 ± 0.11	0.36 ± 0.14
C group	0.35 ± 0.11	0.32 ± 0.10	0.33 ± 0.10	0.30 ± 0.18
N vs. C group	0.50 ± 0.13	0.44 ± 0.12	0.46 ± 0.12	

winter and semi-winter *B. rapa*, respectively (Fig. 4). Subsequently, one individual of each hybrid combination between the hexaploid and *B. rapa* was randomly selected to check pollen fertility and seed set. A significant and positive correlation was found between pollen fertility and seed set ($r = 0.35$, $P < 0.01$). The pollen fertility averaged 69.48 %, ranging from 9.3 to 99.20 %, while the seed set averaged 10.14 seeds per pod, ranging from 0.2 to 28.6 seeds per pod. To verify the hypothesis that individuals of the hybrid between the hexaploid and *B. rapa* with good fertility are new type *B. napus*, we selected 18 individuals of F_1 with pollen fertility of more than 90 % (Fig. 2i) for cytology analysis and found that all individuals had the same number of chromosomes as *B. napus* ($2n = 38$) (Fig. 2j). This indicated that it was easy to select the *B. napus*-like individuals from the progenies between the hexaploid and *B. rapa* based on the pollen fertility.

Genetic variation of new type *B. napus*

To clarify the genetic variation of new type *B. napus*, 76 lines of new type *B. napus* were genotyped with use of SSR markers together with 45 lines of current *B. napus*. The matrix of molecular markers, containing 475 polymorphic alleles of SSR, was used to calculate GD and gene diversity among accessions (Table 1). The average GD between new type *B. napus* and current *B. napus* (0.46 ± 0.12) was larger than that within current *B. napus* (0.33 ± 0.10) and within new type *B. napus* (0.32 ± 0.11), mainly due to more genetic variations in the A subgenome (0.50 ± 0.13) rather than in the C subgenome (0.44 ± 0.12) between new type *B. napus* and current *B. napus* revealed with genome-specific markers (Table 1). Moreover, the new type *B. napus* had higher gene diversity (0.36 ± 0.14) than the current *B. napus* (0.30 ± 0.18) (Table 1), indicating more allele variations in the new type *B. napus* than in the current *B. napus*.

The obvious genetic differences between the new type and current *B. napus* are also supported by the PCA in Fig. 5, where the total variation explained by the first and second principal components were 31.71 and 9.99 %, respectively. The lines of new type *B. napus* clearly separated from the lines of current *B. napus*. In the group of current *B. napus*, the lines of spring and winter *B. napus* were clearly separated, while the semi-winter accessions were dispersed in the middle of winter and spring accessions, in accordance with previous researches (Becker et al. 1995; Qian et al. 2006).

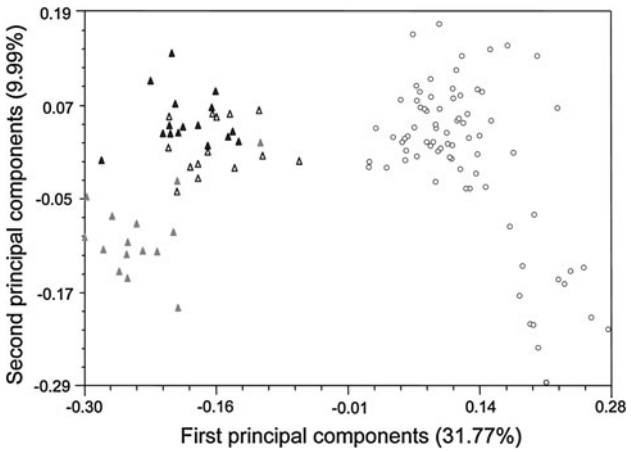

Fig. 5 Association among 76 new type *B. napus* (*white circles*) and 45 current *B. napus* from winter (*black triangles*), spring (*gray triangles*) and semi-winter ecotypes (*open triangles*)

Therefore, those findings indicate that the new type *B. napus* has diverse genetic variation compared with current *B. napus*, especially in the A subgenome, and that the new type *B. napus* can efficiently widen the genetic basis of current *B. napus*.

Discussion

Stability of chromosomal behavior in the hexaploid

Polyploidy is an important process in plant evolution. More than 70 % of angiosperms are polyploid (Wolfe 2001). The natural polyploids usually possess disomic inheritance due to special genes controlling homeologous chromosome pairing and stabilizing the karyotype in natural polyploidy, such as 'ph' gene in wheat (Sánchez-Morán et al. 2001). In contrast, the new resynthesized polyploids are subjected to the challenges of chromosomal structure changes, such as genome rearrangement, epigenetic remodeling and transcript change, resulting in aneuploid progenies (Chen 2010; Huettel et al. 2008; Salmon et al. 2010; Wright et al. 2009). For example, genome instability in resynthesized *B. napus* has been documented (Fujii and Ohmido 2011; Gaeta et al. 2007; Szadkowski et al. 2010, 2011; Xiong et al. 2011), due to a high frequency of homeologous chromosome

pairing between A and C subgenome, which diverged around 0.12 million years ago (Cheung et al. 2009). Until now, the gene of 'PrBn' located in C subgenome of natural *B. napus* was proposed to have main effects on controlling homoeologous pairing between A and C subgenomes in current *B. napus* (Cifuentes et al. 2010; Jenczewski et al. 2003; Liu et al. 2006; Nicolas et al. 2009).

In the present study, the resynthesized hexaploid S0 derived from *B. napus* and *B. oleracea* possessed a relatively stable karyotype, as a majority of its PMCs had normal chromosome segregation in meiosis. But the case of stable karyotype is rare in other resynthesized *Brassica* polyploids, such as the resynthesized hexaploid S0 (AABBCC) derived from *B. carinata* (BBCC) and *B. rapa* with a low euploid frequency of 4.6 % (Tian et al. 2010), and resynthesized *B. napus* betwen *B. oleracea* and *B. rapa* with euploid frequency of 39.74 % in S0 (Wen et al. 2010). Further study is needed to find whether the PrBn-like genes contribute to the stability of the hexaploid with four copies of C-genome in this study.

Moreover, a little improvement of seed set with the increase of the generations of the hexaploid is worth noting in this study, although no artificial selection was employed. It will be possible to develop a novel *Brassica* crop of hexaploid (AACCCC) with normal disomic inheritance if strong selection pressure is employed for fertility and chromosome stability. If so, the development of the hybrid between the hexaploid and *B. rapa* will be stable, and it will be possible to widen the current heterotic patterns of *B. napus* from intervariety heterosis into interspecific or intersubgenomic heterosis, which was demonstrated for strong biomass or seed yield potential (Qian et al. 2003, 2005; Zou et al. 2010).

Widening genetic diversity of *B. napus* with use of *B. rapa*

There are two routine strategies to widen the genetic basis of current *B. napus* with the use of *B. rapa*: resynthesizing *B. napus* between *B. oleracea* and *B. rapa* (Fujii and Ohmido 2011; Girke et al. 2012; Malek et al. 2012 Seyis et al. 2003) and selecting the *B. napus*-like individuals from the progenies between *B. napus* and *B. rapa* (Liu 2000; Qian et al. 2005). However, the complex operation process in the first strategy, such as embryo rescuing and chromosome doubling of the interspecific hybrid, limits the efficiency of resynthesizing *B. napus*. Although the high crossability between *B. napus* and *B. rapa* ensures that the second strategy has been widely applied in the practical breeding program, resulting in the releases of numerous *B. napus* cultivars in China and Japan since 1960s (Liu 2000), it is time-consuming and of low efficiency to select *B. napus*-like individuals from the high generations of interspecific hybridization between *B. napus* and *B. rapa*.

The high crossability between the hexaploid and *B. rapa* in the present study ensured that the genomic components of *B. rapa* could be directly introgressed into *B. napus* without complex operation processes. Therefore, the strategy we propose here is a large-scale, highly efficient method to introduce the genetic variation of *B. rapa* into *B. napus*.

Diverse genetic basis between the new type and current *B. napus* groups was detected in this study, especially in the A subgenome. A possible reason was the introgression of *B. rapa*-specific alleles or genetic alterations during interspecific hybridzation such as chromosome recombination and retrotransposon activations (Gaeta et al. 2007, 2009; Lukens et al. 2006; Song et al. 1995; Xu et al. 2012).

To improve current *B. napus* by exploring the opportunities afforded by *B. rapa*, a recurrent selection population is under construction in our laboratory. The lines of new type *B. napus* are crossed with the male sterile line to develop F_2 segregation populations for fertility, which are sown in an isolation environment for free pollination. The excellent lines pyramiding more genetic components of *B. rapa* will be selected according to their performance and genetic background revealed with molecular markers. The selected fertile lines will be used for breeding, while the open-pollinated seeds from selected sterile lines will be mixed and used for the next cycle.

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