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ORIGINAL PAPER

Broadening the avenue of intersubgenomic heterosis in oilseed *Brassica*

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Abstract Accumulated evidence has shown that each of the three basic *Brassica* genomes (A, B and C) has undergone profound changes in different species, and has led to the concept of the "subgenome". Significant intersubgenomic heterosis was observed in hybrids between traditional *Brassica napus* and first generation lines of new type *B. napus*. The latter were produced by the partial introgression of subgenomic components from different species into *B. napus*. To increase the proportion of exotic subgenomic components and thus achieve stronger heterosis, lines of first generation new type *B. napus* were intercrossed with each other, and subjected to intensive marker-assisted selection to develop the second generation of new type *B. napus*. The second generation showed better agronomic traits and a higher proportion of introgression of subgenomic components than did the first generation. Compared with the commercial hybrid and the hybrids produced with the first generation new type *B. napus*, the novel hybrids showed stronger heterosis for seed yield during the 2 years of field trials. The extent of heterosis showed a significant positive correlation with the introgressed subgenomic components in the parental new type *B. napus*. To increase the content of the exotic subgenomic components further and to allow sustainable breeding of novel lines of new type *B. napus*, we initiated the development of a gene pool for new type *B. napus* that contained a substantial amount of genetic variation in the A^r and C^c genome. We discuss new approaches to broaden the avenue of intersubgenomic heterosis in oilseed *Brassica*.

Introduction

The cytogenetic relationships between *Brassica* species and their genomes were first depicted in the "Triangle of U" (UN 1935), which showed that there are three basic diploid species among *Brassica* crops, *B. rapa* (AA, $2n = 20$), *B. oleracea* (CC, $2n = 18$), and *B. nigra* (BB, $2n = 16$), and three amphidiploids, *B. napus* (AACC, $2n = 38$), *B. juncea* (AABB, $2n = 36$), and *B. carinata* (BBCC, $2n = 34$). The amphidiploids were derived from spontaneous hybridization between the diploids. The Triangle of U has been supported by subsequent research, and the corresponding genomes have been shown to be homologous between the diploid species and their derived amphidiploids (Lagercrantz and Lydiate 1996; Schranz et al. 2006). However, accumulated evidence has shown that the long history of intensive artificial selection in agriculture and rare events of polyploidization have restricted the genetic diversity within each isolated species, and led to genetic differentiation between the amphidiploids and their diploid progenitors (Nishio 2000; Pires et al. 2004). To reflect this differentiation, the concept of the "subgenome" was proposed in *Brassica* with a set of superscripts to distinguish the different subgenomes in different species (Li et al. 2004). For example, the A subgenome from *B. rapa* was designated as A^r, whereas that from *B. napus* was designated as A^n (Fig. 1).

Brassica rapa has been domesticated as an oilseed and vegetable crop in Asia for more than 1,000 years (Liu 2000). The tetraploid species *B. napus* ($A^nA^nC^nC^n$) originated

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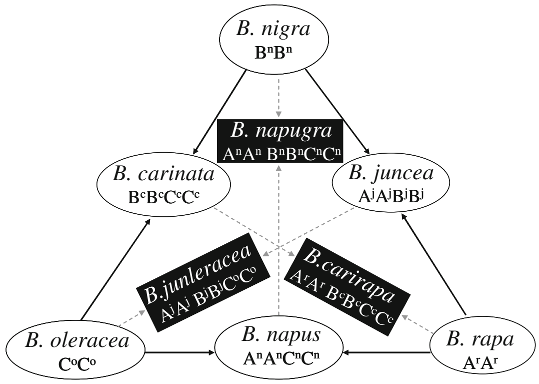

Fig. 1 Diagram modified from the Triangle of U (UN 1935), which shows the relationships among diploid, tetraploid and proposed hexaploidy species of *Brassica* that contain different genomes and subgenomes. *A*, *B*, and *C* represent the three basic genomes in the three basic diploid species, which are located at the three points of the triangle. The three tetraploid species are located between their two parental diploid species. The *superscripts* to each *capital letter* represent the subgenomes that are differentiated in different species (Li et al. 2004). Three artificial hexaploid species synthesized from each pair of tetraploid/diploid species (shown in the *rectangles*) are proposed in "Discussion"

from natural crosses between *B. rapa* (A^rA^r) and *B. oleracea* (C^oC^o) in Europe. It is a relatively young oilseed crop species that has been domesticated and cultivated for only a few hundred years, but it has spread into vast areas of the world including Asia over a few decades (Parkin et al. 1995; UN 1935). *B. napus* was introduced to China in the 1940 s and has been used for interspecific hybridization with oilseed-type landraces of *B. rapa* to breed early maturing, yellow-seeded, and local adaptation since the 1960s. *B. napus* has been the most important oilseed crop because of its superiority over other *Brassica* oilseed crops with respect to yield and seed quality. More recently, *B. napus* has been important for the production of edible oil and feed ingredients, and it has become increasingly important as a raw material for biodiesel in Europe (Sensoz et al. 2000). However, the short history of domestication and cultivation of *B. napus*, and intensive breeding in recent years, have led to a comparatively narrow genetic basis, which limits the potential for exploitation of F_1 hybrid vigor in this species (Becker et al. 1995; Cowling 2007; Rana et al. 2004).

In plants, superior hybrids that give an increased biomass yield can be made, artificially or naturally, by exploiting the enormous genetic diversity between species (Allard 1960). With respect to the production of hybrid oilseeds in China, crosses between Chinese and European cultivars of *B. napus* are more likely to produce an elite hybrid cultivar (Fu 2000). A remarkable level of vegetative heterosis has been observed in the majority of triploid combinations between *B. napus* and *B. rapa*, hence it has been proposed that introgression of the A^r subgenome from *B. rapa* might contribute to heterosis in *B. napus* (Liu et al. 2002; Qian et al. 2003, 2005). On the other hand, a strategy has been developed to exploit interspecific heterosis in rapeseed: *B. napus* ($A^rA^rC^oC^o$) is resynthesized artificially via hybridization between *B. rapa* and *B. oleracea* by embryo rescue (Olsson 1960; Prakash and Raut 1983). However, given that *B. oleracea* is cultivated mainly as a vegetable crop, the resynthesized lines often have low fertility and low seed setting ability. Therefore, usually they are used as a bridging material, and backcrossed several times to natural *B. napus,* to obtain favorable genetic components (Becker et al. 1995; Ren et al. 2000). This approach dilutes the exotic subgenomic components in introgressive lines, which may affect its direct usage in heterosis breeding.

Brassica carinata ($B^cB^cC^cC^c$) has been cultivated in Africa and Asia for thousands of years as an oil and vegetative crop, and has great potential as a genetic resource (Liu 2000). Strong growth vigor has been observed in interspecific hybrids derived from *B. napus* and *B. carinata* (Meng et al. 1998). Therefore, it has been proposed that intersubgenomic heterosis could be used in the breeding of hybrid varieties of oilseed rape by breeding a new type *B. napus*. Firstly, a line with the genome composition $A^rA^rC^nC^n$ or $A^rA^rC^cC^c$ would be generated via the interspecific crossing of *B. napus* with *B. rapa* or *B. rapa* with *B. carinata*, respectively. Secondly, this line would be crossed with traditional *B. napus* to produce $A^rA^nC^nC^n$ or $A^rA^rC^cC^n$ intersubgenomic hybrids (Li et al. 2005; Qian et al. 2005). A series of field trials was conducted for 2–4 years to evaluate the heterosis of the intersubgenomic hybrids with the first generation of new type *B. napus* (new rapeseed first) in China (semi-winter rapeseed growing area), Germany and Denmark (winter rapeseed growing areas), and Canada and Australia (spring rapeseed growing areas with distinctive geographic features). Significant intersubgenomic heterosis was observed in each experiment, and the best intersubgenomic hybrids even surpassed the local control hybrid with respect to seed yield (Li et al. 2006; Qian et al. 2005, 2006, 2007). However, the field trials also showed that the majority of lines of new rapeseed first were not adapted well to local environmental conditions. Severe winters in northern Europe and infection with *Letosphaeria maculans* in Australia and Canada caused heavy losses of yield for many combinations, and meant they required chemical treatments. In addition, only a small proportion of exotic subgenomic components were introgressed into the new rapeseed first lines.

In this paper, we present the progress we have achieved in developing the second generation of new type *B. napus* by pyramiding exotic subgenomic components and performing intensive selection, based on the recombination obtained from intercrosses among lines of new rapeseed first. We demonstrated the increased strength of heterosis in the second generation of intersubgenomic hybrids, as compared with the first generation, and propose the breeding of third generation lines to maximize the potential for the application of intersubgenomic heterosis in oilseed *Brassica*.

Materials and methods

Plant materials and field trials

Thirty-one lines developed by Qian et al. (2003) and Li et al. (2004) as the first generation of new type *B. napus* (new rapeseed first), with introgression of A^r or A^r/C^c genomic components, were used to breed the second generation of new type *B. napus* (Table 1). Four Chinese male sterile lines (MSLs) from Huazhong Agricultural University, China, and two winter and two spring MSLs from the German breeding company NPZ (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG) were used as the female parents, and crossed with selected lines of new type *B. napus* to produce intersubgenomic hybrids.

The hybrids generated with the Chinese MSLs were grown successively in the cities of Daye (2006) and Wuhan (2007), Hubei province, China with three replications in each year. The hybrids generated with the NPZ MSLs were grown successively in Wuhan in 2007 and 2008, with two replications in the first year and three replications in the second year. All field trials were arranged in a random block design. Each plot contained 30 plants in three rows. The local elite commercial hybrid "Huaza6" was used as the hybrid control, and was grown alongside the hybrids in every tenth plot. The seed yield (g/plant) and dry stalk weight (g/plant; weight of the remaining dry stalk with pod debris and branches after harvest) was quantified after harvesting.

Data analysis

The ratios of introgressed subgenomic components (ISC) in new type *B. napus* were evaluated with amplification fragment length polymorphism (AFLP) molecular markers and calculated according to the methods described in Chen et al. (2008). The genetic differences among the different lines of new type *B. napus* were analyzed and clustering analysis was performed with the software NTSYS2.1 (Rohlf 1997). Analysis of variance (ANOVA) on the field trials was conducted using the software SAS 8.0 (SAS Institute 1999).

Table 1 Information about the first generation lines of new type *B. napus* and their parents

Lab code	Field code	Donor parent	ISC[a] (%)	Lab code	Field code	Donor parent	ISC
CHX014	2E467	c2,r6	38	CHX056	2E273	c2,r4	40%
CHX015	2E468	c2,r6	38	CHX057	2E274	c2,r4	38%
CHX016	2E469	c2,r6	35	CHX067	2E366	c2,r4	38%
CHX017	2E470	c2,r6	34	CHX068	2E367	c2,r4	38%
CHX018	2E471	c2,r6	35	CHX070	2E369	c2,r5	40%
CHX019	2E472	c1,r5	31	HAU01	3Q064	r6	
CHX020	2E473	c1,r5	38	HAU04	3Q065	r6	
CHX021	2E474	c1,r5	39	HAU06	3Q066	r1	
CHX022	2E475	c2,r7	36	HAU11	3Q067	r2	
CHX023	2E476	c2,r7	39	HAU14	3Q068	r1	
CHX024	2E477	c2,r7	38				
CHX025	2E478	c2,r2	35	Parents			
CHX026	2E479	c2,r2	34	*B. carinata1*	GZ	c1	Ethiopian landrace
CHX027	2E480	c2,r2	40	*B. carinata2*	101.67	c2	Ethiopian landrace
CHX028	2E481	c2,r2	34	*B. rapa 1*	Denglong	r1	Chinese landrace
CHX050	2E267	c2,r3	37	*B. rapa 2*	Yangyou2	r2	Chinese landrace
CHX051	2E268	c2,r3	30	*B. rapa 3*	Xinghua	r3	Chinese landrace
CHX052	2E269	c2,r3	38	*B. rapa 4*	Shixian	r4	Chinese landrace
CHX053	2E270	c2,r3	35	*B. rapa 5*	Xishui	r5	Chinese landrace
CHX054	2E271	c2,r3	33	*B. rapa 6*	Tianmen	r6	Chinese landrace
CHX055	2E272	c2,r4	39	*B. rapa 7*	Srtyko	r7	Chinese landrace

[a] Ratio of the introgressed subgenomic components. The plant lines that are underlined are the first generation lines that were selected

Results

Selecting elite breeding lines from the first generation of new type *B. napus*

To evaluate the genetic differences and the ratios of introgressed subgenomic components among lines of first generation new type *B. napus*, 26 lines introgressed with A^r/C^c genomic components of differing origin were selected for molecular analysis with 200 AFLP markers (Table 1). These lines of new type *B. napus* contained approximately 30–40% A^r/C^c subgenomic components, which were derived from two cultivars of *B. carinata* and seven of *B. rapa*. The results showed that the index of genetic similarity among different lines varied from 0.47 to 0.96 (Fig. 2a). Eight lines that showed the highest amounts of genetic difference and A^r/C^c introgression were selected as parents to perform intercrosses to pyramid genomic components from the A^r/C^c subgenomes and improve the agronomic traits of new type *B. napus*. In addition, we surveyed previous experimental results (Qian et al. 2006, 2007), and selected five lines of new rapeseed first with a high general combining ability from the 14 lines that were tested in these earlier studies. In total, 13 lines of new rapeseed first with different pedigrees were selected to develop the second generation of new type *B. napus* (new rapeseed second). Twelve of these lines were crossed with each other to produce F_1 hybrids. All of the F_1 lines were crossed with another selected line, HUA04, to produce multiple-cross hybrids (Table 1, Fig. 2b).

Breeding the second generation of new type *B. napus* and evaluating its potential for intersubgenomic heterosis

Twenty multiple intercross combinations were performed with the 13 selected lines of new rapeseed first. The seedlings were screened in the field, and then the genetic background of 2,400 F_2 plants was investigated with 200 AFLP markers. The recombinant progeny showed a wider variation in A^r/C^c content (20–86% compared with 30–40% in the parental generation) (Fig. 3). In addition, plants at different stages of growth showed a large variation in the

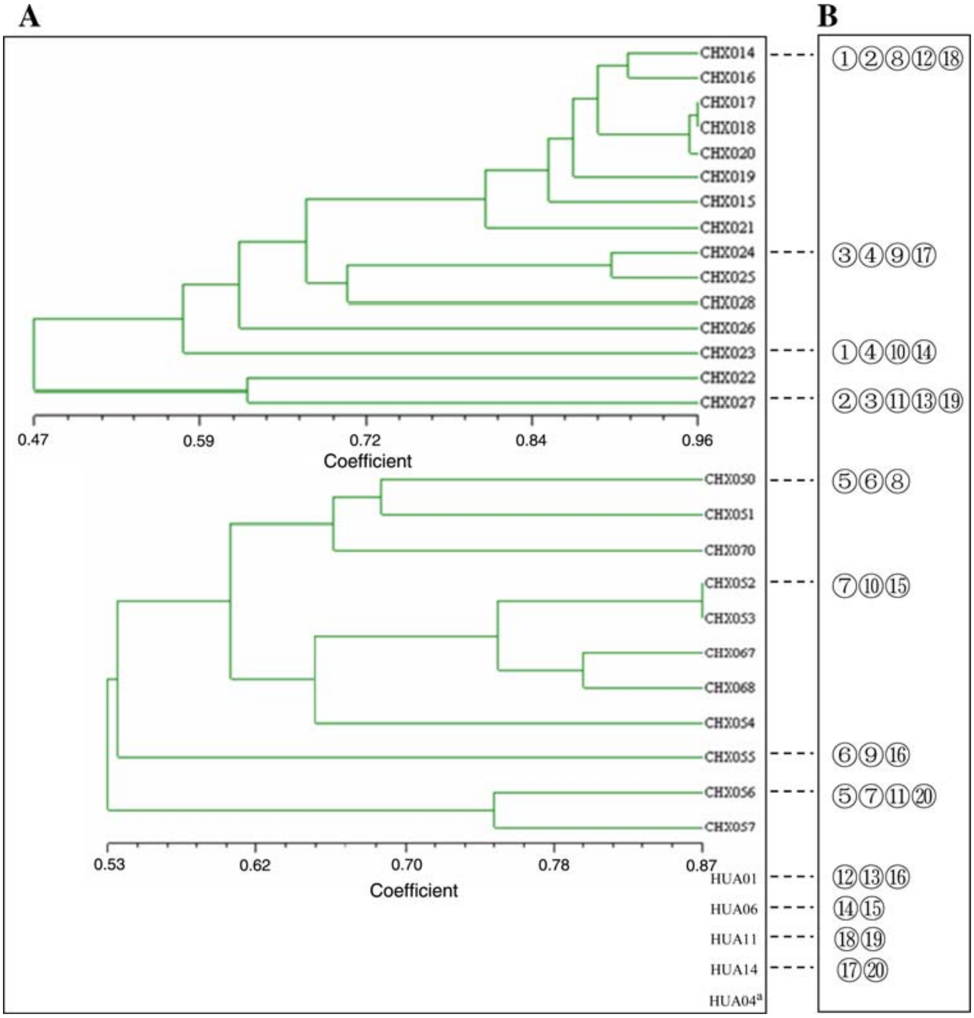

Fig. 2 Genetic evaluation and intercrosses for the selected lines of new rapeseed first. **a** Schematic representation of the genetic similarity among the 26 lines of new rapeseed first. Given that two distinct sets of AFLP markers were used to evaluate the genetic similarity, the 26 lines were separated into two clusters. The five lines under the two clusters represent lines of new rapeseed first with higher general combining ability. These lines were identified in previous experiments and not subjected to cluster analysis. Lines used for intercrosses are shown by a *dashed line* between **a** and **b**. The new rapeseed first line HUA04, shown at the *bottom*, was used as the parent for the multiple crosses. The lines involved in each cross are identified by a pair of *circles* containing the same number

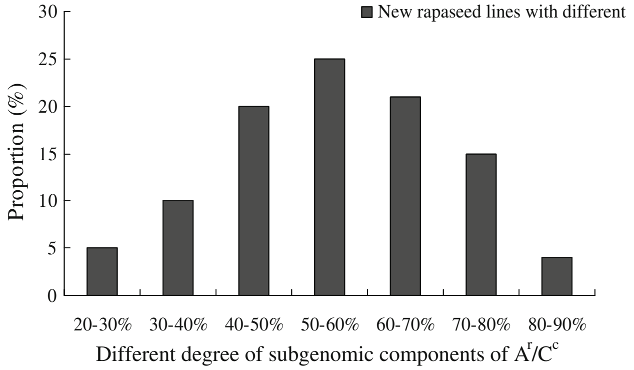

Fig. 3 Proportion of subgenomic components of A^r/C^c as estimated with AFLP markers in 2,400 plants derived from crosses of 20 F_2 populations

field with respect to various traits, such as disease (*Sclerotinia sclerotiorum*) and lodging resistance, flowering time, plant height, seed size, seed number, pod number and seed yield. We harvested one-fifth of the 2,400 plants: those with higher A^r/C^c content and better agronomic characteristics than the remainder. After testing the seed quality (i.e. content of erucic acid, glucosinolate, oil, and protein), approximately 200 F_2 families were grown in different ecological regions of China for further selection and microspore culture. Finally, 80 favorable F_4 plants with improved genetic characteristics and pyramided exotic subgenomic components were bred as the second generation of new type *B. napus* (new rapeseed second).

Two rounds of experiments were carried out to test the heterosis potential of the lines of new rapeseed second. In the first round, 28 lines of new rapeseed second, with a variety of A^r/C^c contents, and five elite lines of new rapeseed first were crossed with four Chinese MSLs. ANOVA analysis for seed yield and other yield related traits showed a significant difference for the interactions between combination and year, but no significant difference was observed for the interactions between combination and replication. Ten percent of intersubgenomic hybrid surpassed the commercial hybrids on seed yield for two years, and those produced with new rapeseed second achieved higher seed yield and biomass yield than that of with new rapeseed first (Fig. 4a). The average correlation coefficients of the introgressed components/seed yield and introgressed components/biomass yield were 0.37 and 0.35, respectively ($p < 0.01$). This demonstrated a significant positive relationship between introgression of the subgenomic components and the heterosis performance of the intersubgenomic hybrids (Fig. 4b).

The results of the first round of experiments were used to select 10 of the 28 lines of new rapeseed second on the basis of their better combining ability. The second round of experiments was carried out with the ten selected lines of new rapeseed second, two elite parental lines of new rapeseed first, and one receptor parent of traditional *B. napus* as male parents. These were crossed with four NPZ MSLs to estimate the wider adaptation of the second generation of intersubgenomic hybrids. Similar results were obtained from the ANOVA and for the performance of the hybrids in terms of yield as those obtained in the first round of experiments (Table 2). More than 30% of combinations surpassed the commercial hybrid, and stronger heterosis appeared when winter MSLs were used as the female parent than when the other MSLs were used. To introduce additional characteristics for local adaptation from the European cultivars into the background of new rapeseed second, two rounds of backcrosses were completed.

Towards for developing the third generation of new type *B. napus*

It is obvious that intersubgenomic heterosis was strengthened after the first generation of new type *B. napus* had been improved genetically by pyramiding exotic subgenomic components and intensive selection. However, a further increase in heterosis appeared difficult to achieve. The genetic resources for new rapeseed second were limited because the original parents that provided exotic genomic

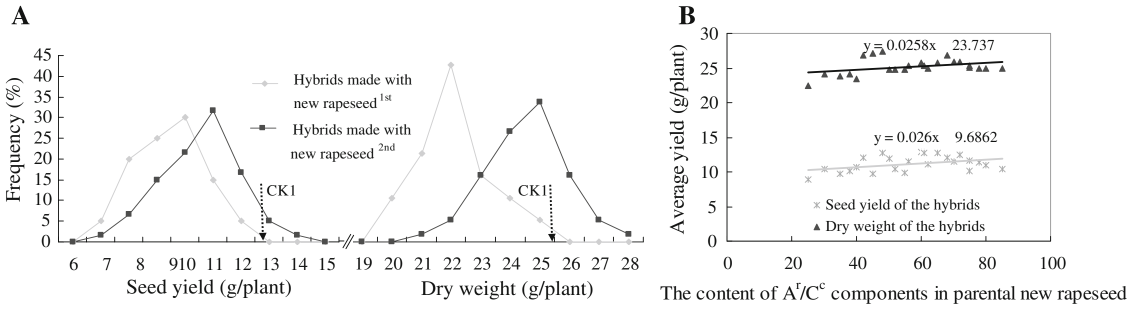

Fig. 4 Average seed yield and dry weight of the intersubgenomic hybrids and the relationship between these parameters and the content of A^r/C^c components in the hybrid parent of new type rapeseed. **a** Distributed frequency of the average seed yield and dry weight of the intersubgenomic hybrids from new rapeseed first and new rapeseed second over 2 years. **b** Positive correlation between the average seed yield and dry weight of the hybrids and the subgenomic components in the hybrid parent of new type rapeseed. The *arrows* labeled CK1 denote the average value for the local commercial hybrid "Huaza 6"

Table 2 Performance of the hybrids produced with the German MSLs in the second experimental round

Male parent	Female parent (MSLs)	Heterosis over control (%)			Heterosis over male parent (%)		
		Max.	Min.	Mean	Max.	Min.	Mean
New rapeseed second	Winter type	46.6 ± 7.3	−30.3 ± 10.1	1.0 ± 2.9	130.1 ± 6.5	−9.2 ± 15.8	59.9 ± 3.2
	Spring type	42.7 ± 4.6	−45.0 ± 3.9	−8.2 ± 5.3	110.8 ± 12.1	−29.1 ± 3.0	43.1 ± 8.1
	All types	46.7 ± 7.3	−45.0 ± 3.9	−3.8 ± 4.1	130.7 ± 6.5	−29.1 ± 3.0	51.7 ± 2.3
New rapeseed first	All types	11.9 ± 10.2	−27.2 ± 4.8	−18.9 ± 8.0			
Traditional *B. napus*	All types	17.3 ± 12.2	−34.2 ± 26.4	−9.8 ± 7.6			

components corresponded to only seven cultivars of *B. rapa* and two of *B. carinata*. Broadening the genetic resources for introgression of A^r/C^c genomic components would assist in the pyramiding of additional exotic components and genetic improvement of new type *B. napus*. The difficulty of interspecific hybridization also limits the breeding potential of novel lines of new type *B. napus*. As a result, we have carried out a plan for developing a third generation of new type *B. napus* by establishing a gene pool that contains substantial amounts of genetic variation in the A^r and C^c subgenomes.

A two-way strategy that we have proposed would increase the efficiency by which diverse subgenomic A^r/C^c components can be introduced into the pool (Fig. 5). In addition, resynthesizing new rapeseed third using the elite lines from the new rapeseed second, which have a high A^r/C^c contents as receptor parents, should assist greatly in the construction of a pool of new type *B. napus* with a high content (>90%) of A^r/C^c subgenomes. Such a project, aimed at breeding elite lines of new rapeseed third, is underway, and the primary results would be reported separately. Obviously, it should be more efficient to develop elite intersubgenomic hybrids by intraspecific crosses between new rapeseed and traditional *B. napus*, than to rely on progeny selected directly from interspecific crossing as hybrid parents.

Discussion

In this present study, we developed a second generation of new type *B. napus* by pyramiding the subgenomic components from lines of first generation new type *B. napus* (new rapeseed first) through recombination and selection. The analysis of intersubgenomic heterosis demonstrated that the genetic basis and heterosis potential of the new rapeseed second lines were increased relatively to those of new rapeseed first. To broaden intersubgenomic heterosis in oilseed *Brassica* further, we initiate the construction of an A^rC^c gene pool that corresponded to a large population of new type *B. napus*, with substantial introgression from A^r and C^c subgenomes. The pool might also expand the genetic diversity of *B. napus*, which has diminished in recent decades (Cowling 2007).

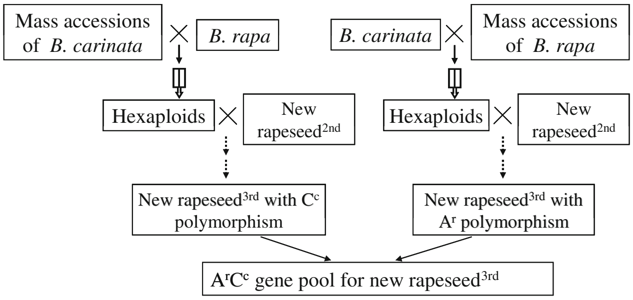

Fig. 5 A strategy to construct an A^rC^c gene pool to produce the third generation of new type *B. napus*. The *bold arrows* represent the process of chromosome doubling, and the arrows with a *dotted tail* represent the production of tetrataploids from pentaploids by successive self-pollination and selection for the plants with 38 chromosomes. In this paper, new rapeseed second and new rapeseed third represent the second and third generation lines of new type *B. napus*, respectively

Currently, we have only exploited the genetic diversity of the A^r genome from *B. rapa* and C^c genome of *B. carinata*. European scientists have introduced new genetic differentiation from the C^o subgenome of *B. oleracea* and A^r of *B. rapa* into resynthesized *B. napus*, which has resulted in substantial biomass heterosis (Bennett et al. 2008; Seyis et al. 2006). However, if agronomic traits of *B. oleracea* could be improved as an oilseed crop before integrating its C genome into resynthesized *B. napus*, direct resynthesis *B. napus* would be a practical way to use intersubgenomic heterosis to improve seed yield. Huge areas are now dedicated to the cultivation of oilseed mustard (*B. juncea*, AABB) in Asia and Ethiopian mustard (*B. carinata*, BBCC) in Africa and the Mediterranean area. Intersubgenomic heterosis would be explored in these crops via a similar strategy to that described here for the creation of new type *B. napus*. Apart from *Brassica* oilseeds, intersubgenomic heterosis might be relevant for other crops, such as wheat and cotton. In the latter, strong heterosis has been reported for hybrids between two species, the upland cotton (*Gossypium hirsutum* AADD) and the sea island cotton (*Gossypium barbadense*, AADD) (Basbag and Gencer 2007).

It has been shown in *Triticale* and other crops that artificial hexapolyploids (or even higher artificial polyploidy) could be used as new seed crops (Siddiqui 1976). We have developed a great number of hexaploid *Brassica* ($A^rA^rB^cB^cC^cC^c$) plants as bridge materials to synthesize new type *B. napus*. Although most of the plants showed various degrees of genetic instability, a few plants have undergone three successive generations of self-pollination with low levels of chromosome loss (unpublished data). This implies that genetically stable hexaploid progeny, with 27 pairs of chromosomes, might be bred as new crops of *Brassica*. One might also expect that at least two other types of *Brassica* hexaploid species, i.e. by crossing *B. napus* with *B. nigra* and crossing *B. juncea* with *B. oleracea*, could be synthesized by interspecific hybridization and selection (personal communication with Dr. Guijun Yuan and Dr. Wallace Cowling; Fig. 1). Several types of hexaploid with different subgenomes, if they are chromosomally stable, could be further crossed with each other, which would result in a strong intersubgenomic heterosis at the hexaploid level in *Brassica* oilseeds.

It seems that the consequences of interspecific crosses extend far beyond the introgression of exotic germplasm. Various genomic changes are induced by interspecific hybridization, for example genome rearrangements (Pontes et al. 2004; Song et al. 1995), parental DNA sequence elimination (Han et al. 2005; Shaked et al. 2001), transposon activation and transposon-induced insertional mutagenesis (Kashkush et al. 2003; Labrador et al. 1999), gene conversion (Kovarik et al. 2005; Wendel et al. 1995), and epigenetic changes (Levy and Feldman 2004; Shaked et al. 2001). It would also be expected that a substantial number of novel genomic alterations might occur in the breeding of new type *B. napus*. A *Copia*-like, transcript-derived fragment was activated in the new type *B. napus* but was absent in most intersubgenomic hybrids (Chen et al. 2008). This suggests that genetic rearrangement might occur when different genomes combine during the process of interspecific hybridization. A proper genetic mapping may provide evidence for such rearrangement events.

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