Extraction of the Constituent Subgenomes of the Natural Allopolyploid Rapeseed (*Brassica napus* L.)

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ABSTRACT As the dynamic nature of progenitor genomes accompanies the speciation by interspecific hybridization, the extraction of the constituent subgenome(s) from a natural allopolyploid species of long history and then restitution of the progenitor(s) provides the unique opportunity to study the genome evolution and interplay. Herein, the A subgenome from the allotetraploid oilseed rape (*Brassica napus* L., AACC) was extracted through inducing the preferential elimination of C-subgenome chromosomes in intertribal crosses and the progenitor *B. rapa* was restituted (RBR). Then by crossing and backcrossing RBR with *B. napus* donor, the C subgenome was *in situ* dissected by adding each of its nine chromosomes to the extracted A subgenome and establishing the whole set of monosonic alien addition lines (MAALs). RBR from spring-type *B. napus* genotype "Oro" expressed a phenotype resembling some type of *B. rapa* never observed before, but showed a winter-type flowering habit. This RBR had weaker growth vigor and suffered more seriously from biotic and abiotic stresses compared with Oro. The phenotypes specific for these MAALs showed the location of the related genes on the particular C-subgenome chromosomes. These MAALs exhibited obviously different frequencies in homeologous pairing and transmission of additional C-subgenome chromosomes, which were associated with the distinct degrees of their relatedness, and even with the possible genetic regulation for meiotic pairing evolved in *B. napus*. Finally, large scaffolds undetermined for sequence assembly of *B. napus* were anchored to specific C-subgenome chromosomes using MAALs.

KEYWORDS allopolyploid; *Brassica napus*; *B. rapa*; alien additional lines; aneuploid

MANY angiosperms including important crops (bread wheat, cotton, oilseed rape, tobacco, etc.) are allopolyploid species that originated from interspecific hybridizations between two or more diploid ancestors followed by chromosome doubling (Ramsey and Schemske 1998; Levin 2003; Otto 2007; Doyle *et al.* 2008; Soltis and Soltis 2012). These allopolyploid crops outcompete their progenitors by their adaptability to a wide range of climatic conditions and higher yield and better quality of targeted products, under human domestication and improvement. To unravel the origin and structure of the component genomes in these crops has been the goal of the extensive genetic research over a long period. The hybridization events leading to the evolutionary origin of these allopolyploids were traditionally elucidated by the artificial synthesis of their counterparts from the crosses between the extant relatives of the presumed progenitors available or by observing the meiotic chromosome pairing in the hybrids between the natural allopolyploid and the possible progenitors (Kihara 1924; Nagaharu 1935). Alternatively, the genome of one progenitor (AA) was readily dissected by separating and adding its own chromosome to the genome of another progenitor (BB) (namely the alien addition lines), from successively backcrossing the natural or synthesized allopolyploid (AABB) to the other progenitor (BB). In another aspect, great efforts were made to develop the whole set of aneuploids for one allopolyploid, which gain or lose one particular chromosome or chromosome arm from the normal complement (nullisomics, monosomics, trisomics, telosomics, etc.) (Sears 1954). These alien additions and aneuploids contributed substantially and excellently to our early knowledge of genome structure and relationship in allopolyploids in tobacco (Clausen and Cameron 1944) and bread wheat (Sears 1954). Recently, the

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sequencing and assembling of the large and repetitive 17-Gb genome of bread wheat was realized by the chromosome-based strategy using the chromosome arms isolated from the complete set of double ditelosomic stocks (Mayer *et al.* 2014).

As the most economically valuable species in the *Brassicaceae* family, the allotetraploid oilseed rape (*Brassica napus* L., $2n = 4x = 38$, AACC) was formed only ~7500 yr ago by the hybridization between the ancestors of two extant diploids *B. rapa* L. ($2n = 2x = 20$, AA) and *B. oleracea* L. ($2n = 2x = 18$, CC) (Nagaharu 1935; Chalhoub *et al.* 2014). The resynthesized and natural *B. napus* has been used as one model to investigate the genomic alterations and interaction at different levels during the allopolyploidization process (Albertin *et al.* 2006; Gaeta *et al.* 2007; Szadkowski *et al.* 2010; Xiong *et al.* 2011; Zhou *et al.* 2011; Cui *et al.* 2012; Chalhoub *et al.* 2014; Zhang *et al.* 2015a). The genome dissection of the extant *B. oleracea* was achieved by adding its individual chromosomes to *B. rapa*, following the synthesis of *B. napus* and backcrossing to *B. rapa* (Quiros *et al.* 1987; McGrath and Quiros 1990; Prakash 2009; Geleta *et al.* 2012; Heneen *et al.* 2012). But the genome dissection for *B. rapa* by such a crossing scheme was rarely reported; likely the crossability between *B. napus* and *B. oleracea* was quite low. For the small and similar size of the chromosomes (Prakash 2009; Heneen *et al.* 2012) and the later development of chromosome-specific cytological markers (Xiong and Pires 2011), the dissection of the A and C genomes in natural *B. napus* was not achieved by the development of the complete set of additions. One nullisomic of *B. napus* with the loss of the C2 chromosome established up to now showed much reduced plant stature and shorter growth period and also the genome-wide change of gene expression (Zhu *et al.* 2015).

As these allopolyploids experienced the evolutionary history of thousands or millions of years, their component genomes diverged from those of the extant descendants of their progenitors (Brenchley *et al.* 2012; Paterson *et al.* 2012; Chalhoub *et al.* 2014; Li *et al.* 2014; Zhang *et al.* 2015b). The extent and pattern of the genomic changes seemed to be affected by age, genome relatedness, and existence or absence of the genetic system regulating the chromosome pairing, such as the *Ph1* loci in wheat. In *B. napus*, abundant homeologous exchanges including crossovers and noncrossovers between two subgenomes were revealed to occur, by the sequence comparisons with extant *B. rapa* and *B. oleracea* (Wang *et al.* 2011b; Liu *et al.* 2014; Chalhoub *et al.* 2014), likely resulting from the close relatedness between the two progenitors (Prakash 2009) and from the absence of the *Ph1*-type system to suppress the homeologous recombination. So the possibility should exist that the exact founder phenotypes of the progenitors for one polyploid might be different from those we studied today, due to evolution of the diploid lineages subsequent to the allopolyploidization event (Buggs *et al.* 2014). Then, the extraction of the constituent subgenome(s) of a given allopolyploid to restitute an independent organism through certain experimental procedures provides the unique opportunity to investigate the genome evolution and interplay. The study of the AABB component extracted from bread wheat (AABBDD) provided novel clues for the modifications in phenotype, karyotype, and gene expression due to a history at the allohexaploid level (Kerber 1964; Zhang *et al.* 2014).

The A subgenome was previously extracted from one *B. napus* cultivar "Oro" and the ancestral *B. rapa* was restituted (RBR Oro) by inducing the preferential loss of the C-genome chromosomes in intertribal crosses with *Isatis indigotica* ($2n = 14$) (Tu *et al.* 2010). The cultivar was likely representative of one origin event for *B. napus*, as it was bred from the landrace "Liho" in Germany and kept the commonest type of plastid haplotypes among a wide range of *B. napus* accessions (Allender and King 2010). In this study, Oro was successively backcrossed to the RBR Oro to dissect the C subgenome in the A-subgenome background of the same origin and to establish a complete set of monosomic alien addition lines (MAALs) with one of its nine chromosomes. With the C-subgenome chromosome-specific gene markers designed from the draft sequences of *B. napus* (Chalhoub *et al.* 2014), the whole set of additions was distinguished and established, which inversely helped to anchor some undetermined scaffolds to specific chromosomes. The successful *in situ* dissection of the C subgenome in natural *B. napus* not only provides the unique genetic stock for analyzing its structure and function but also advances our insights into the genome change and interplay during the evolution of this allotetraploid species. In addition, the feasibility of the restitution of ancestral *B. rapa* was further confirmed in the intertribal crosses between different *B. napus* genotypes and another crucifer *Crambe abyssinica*, and the cytological process of the successive elimination of C-subgenome chromosomes was further elucidated.

Materials and Methods

Plant materials

To investigate the cytological process of extracting the A subgenome from natural allotetraploid *B. napus* ($2n = 38$, AACC), the elite cultivar "Zhongshuang 11" was pollinated by another crucifer *C. abyssinica* ($2n = 90$) to induce the preferential elimination of the C-subgenome chromosomes (Figure 1). Out of 92 seeds harvested from ~10,000 flowers pollinated, only 36 seeds germinated and produced F_1 plants that showed a phenotype quite similar to the female parent. Among 36 F_1 plants, 32 were found to keep the same chromosome number as the *B. napus* parent and were not used for further experiment. The remaining four F_1 plants were identified to have $2n = 29$ and were pollinated again by *C. abyssinica*, and 33 backcross (BC_1) plantlets were obtained from more than >2000 crosses by embryo rescue on MS agar medium (Murashige and Skoog 1962) without hormones. These plantlets were propagated by subculturing the young buds on MS medium with 1.5 mg/liter^{-1} 6-benzyl aminopurine (6-BA), and 0.25 mg/liter^{-1} α-naphthalenacetic acid (NAA), to produce enough plants for study. Among BC_1

plants, 30 had $2n = 30$–44 and showed the variations in the phenotype. The other three BC_1 plants ($2n = 22$, 23, and 26) gave the *B. rapa*-type morphology. After the plant with $2n = 22$ was self-pollinated with artificial assistance, the plants with $2n = 20$ (AA) were derived and designated as RBR ZS11, the restituted *B. rapa* from Zhongshuang 11.

Previously, the diploid ancestor *B. rapa* ($2n = 20$, genome AA) was extracted from the allotetraploid *B. napus* cv. Oro ($2n = 38$, AACC), after being repeatedly pollinated by another crucifer *I. indigotica* ($2n = 14$, II) (Tu *et al.* 2010). After the restituted *B. rapa* genotype (RBR Oro) was crossed with Oro as female, the triploid hybrid ($2n = 29$, AAC) was readily obtained and pollinated by RBR Oro to generate BC_1 plants ($2n = 20$–29). Those BC_1 plants with different C-subgenome chromosomes were pollinated again by RBR Oro to produce BC_2 plants ($2n = 20$–26), and several BC_2 plants ($2n = 22$) that were found to carry chromosome C4 were pollinated further by RBR Oro. Both BC_2 and BC_3 plants ($2n = 20$–22) were screened to establish MAALs for different C-subgenome chromosomes ($2n = 21$, AA + $1C_{1–9}$), by both cytological and molecular selections (Figure 3). The cytological numerical designations of the nine C-subgenome chromosomes followed the internationally adopted system (Wang *et al.* 2011a; Xiong and Pires 2011; Chalhoub *et al.* 2014).

Because the growth of RBR Oro and the hybrid progenies was severely impeded under humid conditions during the winter season in the experimental field in Wuhan, these plants were grown in pots in an unheated glasshouse before flowering and later transferred to the field to produce seeds during the spring season.

Morphology, cytology, and pollen viability

Morphological characteristics of the complete set of MAALs were documented and compared to those of Oro and RBR Oro. To determine the chromosome numbers of the hybrids between Oro and RBR Oro and backcrossing progenies of successive generations, the ovaries from young flower buds were collected and treated with 2 mM 8-hydroxyquinoline for 3 hr at ~20° and then fixed in Carnoy solution (3:1 ethanol: glacial acetic acid, v/v) and stored at −20° for use. For meiotic analyses, young flower buds from fully blooming plants were collected and fixed in Carnoy solution for 12 hr and then transferred to fresh liquid three times and stored at −20°. Cytological observation was followed according to the method of Li *et al.* (1995). The percentage of stainable pollen grains stained with 1% acetocarmine of >500 pollens per MAAL line was calculated to determine pollen viability.

Probe labeling, genomic in situ hybridization, and FISH analysis

The plasmid DNA of BAC BoB014O06 specific for the C genome of *B. oleracea* (provided by Susan J. Armstrong, University of Birmingham, Birmingham, UK) and genomic DNA of *C. abyssinica* were labeled with biotin-11-deoxycytidine triphosphate by random priming using the BioPrime DNA Labeling System kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The genomic DNA of *B. napus* Zhongshuang 11 was fragmented in boiling water and used as a block. To identify specific C-subgenome chromosomes, four repetitive DNA sequences: 5S ribosomal DNA (rDNA), 45S rDNA, CentBr1, and CentBr2 were labeled with digoxigenin-11-deoxyuridine triphosphate (Roche, Basel, Switzerland) by random priming using the BioPrime Array CGH Genomic Labeling System kit according to the manufacturer's protocol. Multiple target FISHs with these probes were performed to identify certain MAALs according to the work of Xiong and Pires (2011). Slide preparations were carried out mainly according to the methods of Zhong *et al.* (1996) and the procedures of BAC-FISH analyses following the procedure of Cui *et al.* (2012) with slight modification to reduce the washing time to 8 min in 0.1 × SSC with 20% deionized formamide at 40°. Before a second round of FISH, slides were washed for 10 min in 2 × SSC with 50% deionized formamide at 42° to clear former hybridized probe.

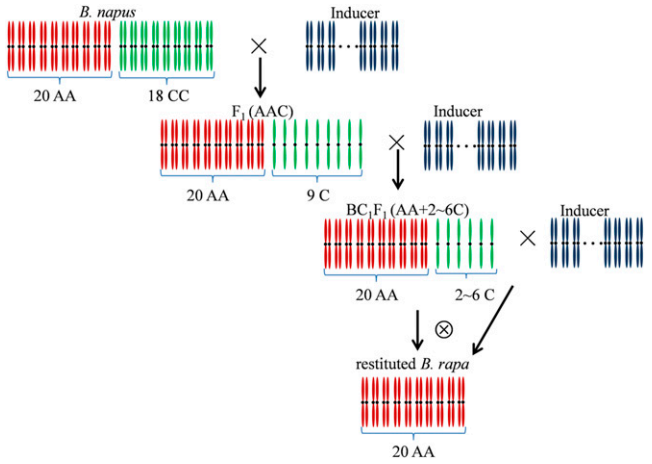

Figure 1 Crossing and cytological process of A-subgenome extraction and *B. rapa* restitution from *B. napus* induced by intertribal hybridizations. The inclusion of the complete A subgenome in F_1 hybrid and backcrossing progenies was traced and shown by cytological observations (see Figure 2).

Images from FISH were taken using a computer-assisted fluorescence microscope with a CCD camera (Axio Scope A1, Zeiss, Oberkochen, Germany). Photographs were manipulated by Adobe Photoshop 7.0 software to adjust contrast and brightness and change the background to black.

Data statistics

The significance of data statistics, including *t* test, Pearson's correlation coefficient as well as χ^2 test, was determined by the data analysis function of Microsoft Excel and the R project.

Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism (AFLP) analysis was performed for the hybrids, RBR ZS11, and parents according to the protocol of Vos *et al.* (1995). A total of 50 ng DNA per sample was digested by the restriction endonucleases

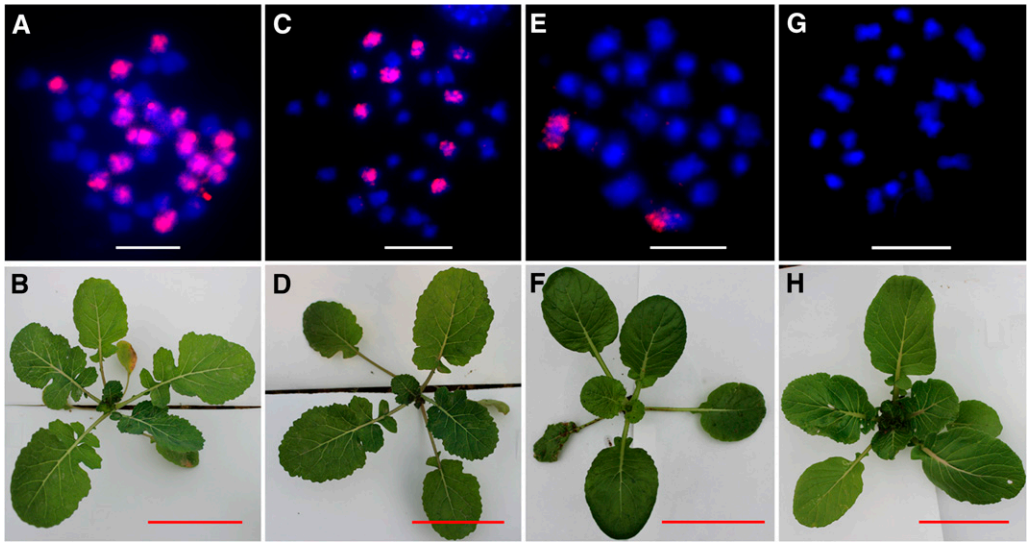

Figure 2 Chromosome complement and phenotype of plants from *B. napus* to RBR. Blue indicates DAPI staining of chromosomes and red indicates labeled BAC BoB014O06 probe specifically for the C genome (Bar, 10 μm). The phenotype of young plants is shown. Bar, 10 cm. (A and B) One ovary cell ($2n$ = 38) with 20 A-subgenome chromosomes (blue) and 18 C-subgenome chromosomes (red) (A) from *B. napus* cv. Zhongshuang 11 (B). (C and D) One ovary cell ($2n$ = 29) with nine C-subgenome chromosomes (red) and 20 A-subgenome chromosomes (blue) (C) from F_1 hybrid (D). No signals for the *C. abyssinica* genomic DNA probe (green) are detected. (E and F) One ovary cell ($2n$ = 22) with 20 A-subgenome chromosomes (blue) and 2 C-subgenome chromosomes (red) (E) from one BC_1F_1 plant (F). (G and H) One ovary cell ($2n$ = 20) with no red signals on all chromosomes (G) from RBR (H).

*Eco*RI and *Mse*I (Thermo Fisher Scientific, Waltham, MA), then ligated with *Eco*RI and *Mse*I adapters. Two steps of PCR (preselective PCR and selective PCR) were performed to amplify the ligation production. In total, 57 primers were selected and used for AFLP fingerprint analysis.

Design of C-subgenome chromosome-specific gene markers and PCR amplification

Based on the recently released genome data of *B. napus* (Chalhoub *et al.* 2014), 117 genes, anchored in one of nine C-subgenome chromosomes and including obvious sequence variations compared to their respective A subgenome homeologs, were chosen for primer design. Primers were designed by Oligo 7 software based on those sequences with variations and then blasted to the *B. napus* genome. Those primers with more than one potential binding site were discarded. Young leaves were collected to extract DNA according to the CTAB method. PCR reactions (10 μl) contained 1× Taq buffer, 2 mM $MgCl_2$, 2.5 mM deoxy-ribonucleoside triphosphatesans, 5 μM forward and reverse primer, 0.35 units Taq DNA polymerase, and 50 ng genomic DNA. DNA fragments were amplified using an initial 5-min denaturation at 94° followed by 30 cycles (94° for 45 sec, 53° −57° for 30 sec, and 72° for 45 sec), and a final 10-min elongation step at 72°. Finally, PCR products were checked by 1% agarose gels.

Supplemental Material, File S1 contains Table S1, Table S2, Table S3, and the supplemental data about the detailed description of morphological characteristics of MAALs. Table S1 contains detailed information of C-subgenome chromosome-specific gene markers. Table S2 contains the number of plants used to determine the transmission rates per MAAL. Table S3 contains anchored scaffolds determined by the set of MAALs and those anchored specific gene markers.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Extraction of the A subgenome from B. napus by inducing the elimination of C-subgenome chromosomes

Although the A subgenome was extracted from the allotetraploid *B. napus* ($2n$ = 38, AACC) after the C-genome chromosomes were successively and completely eliminated by pollination with another crucifer *I. indigotica* ($2n$ = 14) (Tu *et al.* 2010), the cytological process related to the biased loss and the pervasiveness of this method needed to be elucidated in detail. Herein, *B. napus* cultivar Zhongshuang 11, the first genotype (Figure 2, A and B) that was selected for genome sequencing in China, was extensively pollinated by the crucifer *C. abyssinica* ($2n$ = 90), which flowered at a similar time to *B. napus* and had a longer flowering duration compared with *I. indigotica*. Four F_1 hybrids from ~10,000 pollinations were identified to have $2n$ = 29, but showed the *B. napus*-type morphology (Figure 2, C and D). They contained 20 chromosomes from A-subgenome and 9 C-subgenome chromosomes as revealed by FISH with C-subgenome-specific probe (Figure 2C), but no chromosomes and chromosomal fragments from *C. abyssinica* were detected by genomic *in situ* hybridization (GISH) analyses (Figure S1, A1–A3). Additionally, FISH with C-subgenome-specific probe was also performed to check the specificity of this probe in root-tip cells of *C. abyssinica* (Figure S1, C1–C3). Further investigations with gene markers specific for individual C-subgenome chromosomes confirmed that these 9 C-subgenome chromosomes covered all 9 chromosomes of the

Table 1 AFLP analysis of intertribal hybrids and RBR from *B. napus* Zhongshuang 11 × *C. abyssinica* cross

Plant code	Specific bands for *C. abyssinica* (%)	Novel bands for two parents (%)	Deleted bands for *B. napus* (%)
F-1[a]	25 (2.56)	65 (6.76)	109 (11.33)
F-2[a]	27 (2.76)	61 (6.34)	132 (13.72)
F-3[a]	34 (3.48)	72 (7.48)	142 (14.76)
F-4[a]	30 (3.07)	60 (6.24)	124 (12.89)
Average	29 (2.97)	64.5 (6.71)	126.75 (13.18)
RBR ZS 11	16 (1.64)	129 (13.41)	332 (34.51)

[a] Intertribal hybrids.

C-subgenome (data not shown), indicating their chromosome component of AAC with the loss of one copy of the C subgenome from the complement of *B. napus*. Its chromosomes in pollen mother cells (PMCs) showed two kinds of pairing configurations, 1III + 9II + 8I (64.4%, 58/90) and 10II + 9I (35.6%, 32/90). After these hybrids (AAC) were pollinated again by *C. abyssinica* to induce more loss of C-subgenome chromosomes, three BC_1 plants exhibited a *B. rapa*-type phenotype and had lower chromosome number ($2n$ = 22, 23, and 26), including 20 A-subgenome chromosomes and additional C-subgenome chromosomes. Among the self-pollination progenies from one BC_1 plant with $2n$ = 22 (Figure 2, E and F), >100 plants with 20 A-subgenome chromosomes appeared and gave the *B. rapa*-like phenotype (Figure 2, G and H), which were the restituted *B. rapa* from Zhongshuang 11 (RBR ZS11). In PMCs of RBR ZS11, normal chromosomes pairing as 10 bivalents and 10:10 segregation were performed. Then the flowers had well-developed anthers full of highly stainable pollen grains (90.0 ± 2.3%), which was comparable to donor *B. napus* (92.8 ± 2.3%) (*t* test, $P > 0.05$). However, their seed sets by selfing pollination were quite low (1.92 seeds/pod), but highly enhanced by bud pollination (6.59 seeds/pod), indicating a barrier of selfing seed setting or the regain of self-incompatibility for *B. rapa*. By AFLP analysis with 57 pairs of primers, the four hybrids and RBR ZS11 lost 11.33–14.76% and 34.51% of the *B. napus*-specific fragments, respectively, and contained 2.56–3.48% and 1.64% of the *C. abyssinica*-specific fragments (Table 1). Additionally, 6.24–7.48% and 13.41% of fragments novel for two parents were present in the hybrids and RBR ZS11, respectively.

Taken together, the present and previous results (Tu *et al.* 2010) manifested the feasibility to extract the complete A subgenome and restitute the ancestral *B. rapa* genotype from natural *B. napus* through inducing successive loss of C-subgenome chromosomes, after two or three rounds of pollinations by other crucifers of different genera/tribes (Figure 1). The completeness of the extracted A subgenome was shown not only by the karyotype ($2n$ = 20) and normal meiotic division of RBR Oro and RBR ZS11, but also by the construction of a linkage map including 10 linkage groups using the F_2 population from the cross between RBR Oro and the sequenced *B. rapa* genotype "Chiifu" (Zhang Dawei and Li zaiyun, unpublished data).

Additions of C-subgenome chromosomes to RBR

To dissect the C subgenome of natural allotetraploid *B. napus*, *B. napus* cv. Oro was pollinated by RBR Oro (Tu *et al.* 2010), and the hybrid seeds were easily produced (Figure 3). The F_1 hybrid plants were confirmed to be triploid ($2n$ = 29, AAC) and their chromosome pairings were as follows: 1III + 9II + 8I (65.06%), 10II + 9I (26.51%), 2III + 8II + 7I (6.02%), and 1IV + 8II + 9I (2.41%). Then, more than 20 hybrids were pollinated again by RBR Oro to generate BC_1 plants. Out of 201 BC_1 plants ($2n$ = 20–29) with chromosome numbers determined in the young ovary cells (Table 2), 13 (6.5%) had $2n$ = 20 for euploid *B. rapa*, 12 (6.0%) had $2n$ = 21 for putative monosomic alien addition lines (MAALs), and the remaining kept the higher chromosome number (Table 2). Unfortunately, the plants of these putative MAALs grew weakly and failed to produce seeds due to high sensitivity to some diseases in the field, and only one matured and gave the seeds after pollination by RBR Oro, which was later confirmed to carry chromosome C8 (AA + C8). Nine plants had $2n$ = 28 and three plants still kept one whole set of C genome ($2n$ = 29, AAC), and the remaining plants had $2n$ = 22–27 (Table 2). Then about 90 BC_1 plants with $2n$ = 21–27 were pollinated by RBR Oro to generate BC_2 progenies. Among 445 BC_2 plants observed ($2n$ = 20–26) (Table 2), 237 (53.3%) were of RBR Oro-type ($2n$ = 20) and 117 (26.3%) were MAALs with one of nine C-subgenome chromosomes except C4. Out of the other BC_2 plants with two or more C-genome chromosomes, several double MAALS (DMAALs) harboring C4 and another C-subgenome chromosome were pollinated again by RBR Oro, and MAALs with C4 were selected among BC_3 progenies from two DMAALs (C4 + C3 and C4 + C7) (Figure 3). Because of severe low selfing seed sets for most additional lines, all MAALs were preserved by backcrossing to RBR Oro.

Specific gene markers of individual C-subgenome chromosomes

Genome sequences of *B. napus* genotype "Darmor" (Chalhoub *et al.* 2014) gave references for chromosome-specific genes of the C subgenome to identify the complete set of MAALs. Totally, 117 specific genes or gene sequences distributed on nine C-subgenome chromosomes were selected to design primers to determine their identities in each MAAL. Among 117 gene markers, 49 pairs of primers (Table S1) gave rise to specific fragments of the C subgenome after being screened in four *B. napus* cultivars (Oro, Zhongshuang 11, Huashuang 3, and Zhongyou 821), four *B. rapa* genotypes (RBR Oro, RBR ZS11, and Chiifu, Baiyou 1), and four BC_1

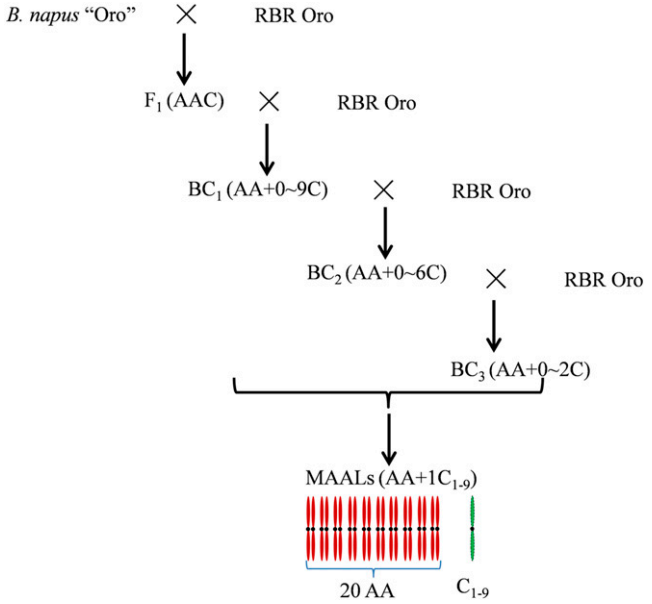

Figure 3 Development of MAALs and dissection of the C subgenome in the background of RBR.

plants carrying different numbers of C-subgenome chromosomes ($2n$ = 21, 23, 24, and 26). The remaining primers generated either no amplified products or no polymorphic products among these screened materials, probably attributed to improper designed primers and the difference of genomic backgrounds between Darmor and Oro. The number of specific markers for each C-subgenome chromosome was four to seven, which were located on two arms of one assembled chromosome in sequencing. Those markers along every C-subgenome chromosome presented perfectly consistent results, when used to identify and classify the complete set of MAALs designated as AAC1–AAC9 (Figure 4).

Additionally, one plant with $2n$ = 20 also presented all the amplified products of specific primers for chromosome C1, suggesting the monosomic substitution line of *B. rapa*, which was confirmed by the FISH analysis with C-genome-specific BAC probe. One plant with $2n$ = 21 showed the amplified products of all C1-specific markers and some C8-specific markers, which included one intact additional C1 chromosome and one A-subgenome chromosome with one segment translocated from C8. These two plants were not further investigated, for not being MAALs.

Integrity of C-subgenome chromosomes in MAALs

To confirm the integrity of C-subgenome chromosomes in MAALs, FISH analyses with BAC BoB014O06 as probe (Cui *et al.* 2012; Zhu *et al.* 2015) were performed on several selected plants from each MAAL (Figure 5A). It was manifested that all plants observed carried one complete C-genome chromosome, which was in line with the results from molecular makers above. Then, according to the method of Xiong and Pires (2011), a dual FISH with BAC BoB014O06 and 5S rDNA as probes was applied to MAALs with C4 (Figure 5B). Two

Table 2 Distributions of chromosome numbers among BC_1 and BC_2 plants from the hybrids ($2n$ = 29, AAC) between Oro and RBR Oro backcrossed to RBR Oro

	Chromosome nos. ($2n$)										
Generations	20	21	22	23	24	25	26	27	28	29	Total
BC_1	13	12	29	30	26	27	28	24	9	3	201
BC_2	237	117	49	24	8	9	1	0	—	—	445

continuous rounds of multiple target FISH with repeated sequences CentBr1, CentBr2, 45S rDNA, and BAC BoB014O06 as probes were applied to MAALs with C7 (Figure 5, C and D) and C8 (Figure 5, E and F), to prove the reliability of these specific gene markers. In addition, all or some of the five probes above were applied to detect the other MAALs and showed consistent results of molecular markers (Table 3). Taken together, the results from these cytological and gene-specific markers provided robust evidence for the complete set of MAALs with each C-subgenome chromosome in the background of the A subgenome extracted from the same natural *B. napus*, *i.e.*, the *in situ* dissection of the C subgenome.

Chromosome pairing of MAALs

By FISH with the probe BAC BoB014O06 specific for the C genome, the chromosome pairings for each MAAL were observed in 97–123 PMCs at diakinesis, respectively (Table 4). Totally, the majority of PMCs (68.91–87.13%) had the pairing configuration with $10II^{AA} + 1I^{C}$ (Figure 6B) and the minority (10.89–26.05%) showed the pairing with $9II^{AA} + 1III^{AAC}$ (Figure 6C). Additionally, in a small part of PMCs (0.81–6.03%) from all MAALs, one autosyndetic quadrivalent was formed by A-subgenome chromosomes, resulting in the pairing with $8II^{AA} + 1I^{C} + 1IV^{AAAA}$ (Figure 6D). But the quadrivalent was not observed in RBR Oro (Figure 6A). AAC1 and AAC2 showed the two highest percentages of PMCs (26.05% and 24.14%) with the allosyndetic trivalent, likely due to the high homeology between A1 and C1 and A2 and C2. They also had the highest percentages of PMCs (6.03% and 5.04%) with one A-subgenome quadrivalent. Notably, the percentage of PMCs (10.89%) with allosyndetic trivalent was lowest in AAC9. AAC4 also showed comparably low frequency of allosyndetic trivalent (14.42%) (χ^2 test, $P > 0.05$). The other MAALs had 17.48–23.89% PMCs with $9II^{AA} + 1III^{AAC}$ and 0.81–3.09% PMCs with $8II^{AA} + 1I^{C} + 1IV^{AAAA}$ (Table 4). The average pairing per cell for each MAAL is summarized in Table 4.

Transmission rate of additional chromosomes through male and female gametes

To assess the transmission rates of additional C-subgenome chromosomes, 95–131 (from two plants of MAAL per chromosome except for AAC9 with only one plant obtained in BC_2) and 66–93 (from one plant of MAAL per chromosome) plants from reciprocal crosses between each MAAL and RBR Oro were obtained to analyze the female and male transmission

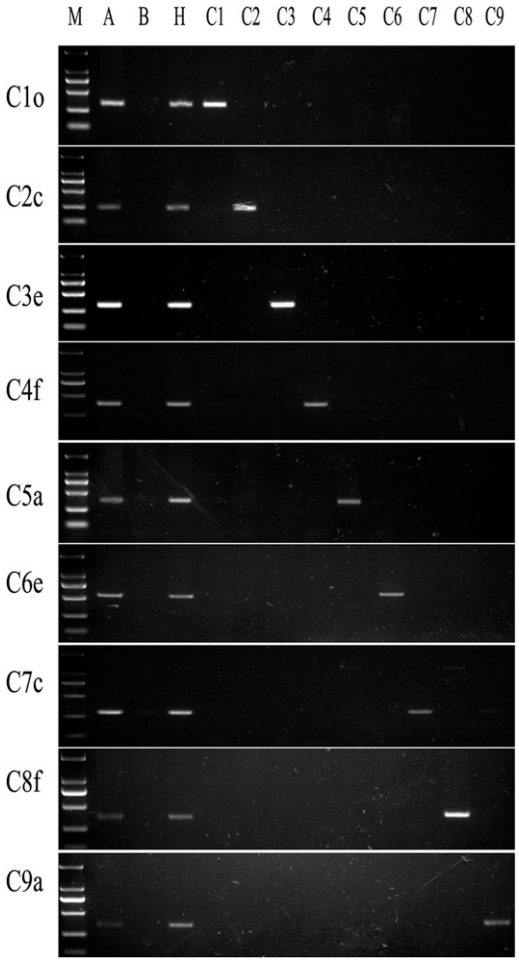

Figure 4 C-subgenome chromosome-specific gene markers for the complete set of MAALs. A, *B. napus* cv. Oro; B, RBR Oro; H, hybrid of Oro and RBR Oro; and C1–C9, nine MAALS. The sizes of marker ladder from top to bottom are 2000, 1000, 750, 500, 250, and 100 bp, respectively.

rates, respectively (Table S2). Then four gene markers distributed along both arms per C-subgenome chromosome were selected to determine its existence in the progeny plants. For female transmission of each C-subgenome chromosome in MAALs, the rates varied from 7.9% for AAC9 to 33.0% for AAC1 (Table 4), averaging 20.5%. For male transmission (except AAC9), the rates were from 3.2% for AAC3 to 13.3% for AAC1 (Table 4), averaging 7.2%, much lower than the female transmission rate. The male transmission of C9 was not detected, likely due to the lowest pollen fertility of AAC9 (69.0 ± 0.5%) and the limited number of progenies observed. The C1 chromosome showed the highest transmission rates by both male (13.3%) and female gametes (33.0%). In AAC4 and AAC9, the transmission rates (8.3 and 7.8%) for female were significantly lower than those of the others (χ^2 test, $P < 0.05$). In AAC4, the transmission rate for male (8.8%) was slightly higher than that for female (8.3%). Interestingly, the female transmission rate was highly correlated with pollen viability of MAALs ($R^2 = 0.81, P = 8 \times 10^{-4}$), but the correlation between male transmission rate and pollen viability was not as significant ($R^2 = 0.29, P > 0.05$), implying the weaker competitiveness of aneuploidy male gametes in the process of fertilization. In several AAC2 plants and progenies, two markers, C2b and C2c, were missing, indicating the addition of the incomplete C2 chromosome and its inheritance to progeny.

Table 3 Cytological markers for the complete set of MAALs

MAALs	Probes and signals			
AAC1	**BAC BoB014O06**	**CentBr1**	**CentBr2**	*5S rDNA*
AAC2	**BAC BoB014O06**	**CentBr1**	*CentBr2*	*45S rDNA*
AAC3	**BAC BoB014O06**	*CentBr1*	**CentBr2**	*45S rDNA*
AAC4	**BAC BoB014O06**	**5S rDNA**		
AAC5	**BAC BoB014O06**	**CentBr1**	*CentBr2*	*45S rDNA*
AAC6	**BAC BoB014O06**	**CentBr1**	*CentBr2*	*45S rDNA*
AAC7	**BAC BoB014O06**	**CentBr1**	**45S rDNA**	
AAC8	**BAC BoB014O06**	*CentBr1*	**CentBr2**	**45S rDNA**
AAC9	**BAC BoB014O06**	*CentBr1*	*45S rDNA*	

Boldface type denotes that FISH signal with this probe is detected in given MAAL and italics denote no signal for this probe.

Anchoring undetermined scaffolds of B. napus by MAALs

As ~13% of scaffolds remained undetermined to certain chromosomes in the sequenced genome of *B. napus* (Chalhoub *et al.* 2014), this complete set of MAALs would help to anchor the elusive C-subgenome scaffolds to specific chromosomes. Therefore, 22 nonanchored C-subgenome scaffolds, ranging from 0.049 to 1.231 Mb and totally covering >10 Mb as well as assembling 1097 predicted genes, were selected to be anchored to C-subgenome chromosomes, based on presence/absence of amplified products of designed primers in nine MAALs. In total, 108 gene markers, corresponding to 108 anchored genes assembled to these scaffolds, were picked out. DNA extracted from three plants per genotype was mixed to prepare a pool. After PCR amplification, 45 gene markers from all 22 scaffolds (Table S3) were uniquely amplified in a single MAAL, resulting in the successful anchoring of these scaffolds to eight chromosomes of the C subgenome except C1.

Morphology of RBR and MAALs

As the exact progenitors of *B. napus* cv. Oro were unknown, RBR Oro would reflect largely the phenotype of the *B. rapa* ancestor several thousand years ago. While Oro, the first cultivar with low content of erucic acid in the world, which was selected from the local variety "Liho" in Germany, was a spring oilseed type, RBR Oro showed a flowering habit similar to the winter type of *B. rapa* (Guo *et al.* 2014). When planted in October in Wuhan, the young plants produced the procumbent leaves (Figure 7) with trichomes and many accessory buds under the low temperature, not observed in Oro (Figure S2). Similar phenotypes were also observed in RBR ZS11. The leaves and whole plants of RBR were slightly green, not so deep as those of their parental *B. napus*, and the phenotype and growth habitat were different from those *B. rapa* genotypes in our hands. The plants of RBR Oro flowered and matured earlier than Oro, and showed much lower

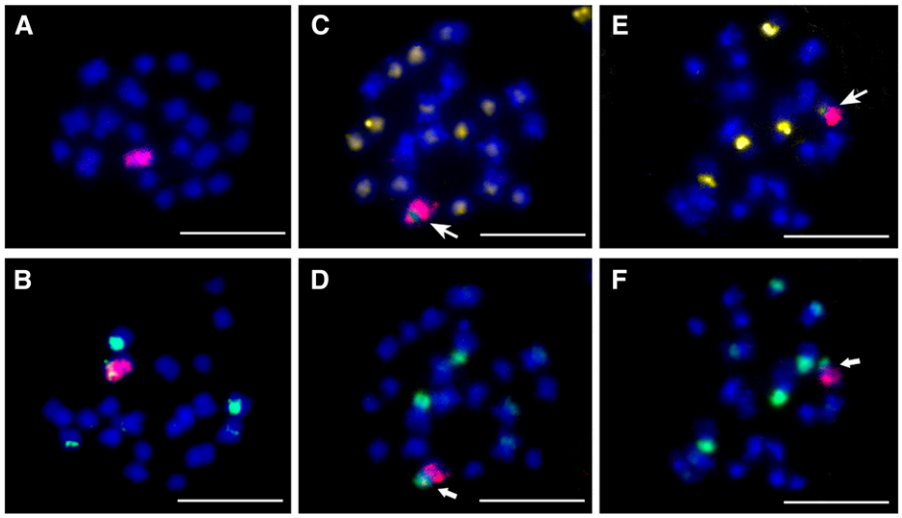

Figure 5 BAC-FISH analyses of mitotic cells in MAALs. Blue indicates DAPI staining and red indicates labeled BAC BoB014O06 probe. (A) One mitotic cell for MAAL with 20 A-subgenome chromosomes (blue) and one C-subgenome chromosome (red). (B) One mitotic metaphase of AAC4 in which the chromosome C4 (red) harbors 5S rDNA locus (green). (C–F) Identification of AAC7 and AAC8 by applying two rounds of multiple target FISH. (C and D) One mitotic cell of AAC7. The chromosome C7 (red) gives the signals from CentBr1 probe (yellow, big arrow) in the first round FISH (C) and from 45S rDNA probe (green, solid arrow) in the second round FISH (D). (E and F) One mitotic cell of AAC8. The alien chromosome (red) is labeled with CentBr2 (yellow, big arrow) (E) and 45S rDNA probe (green, solid arrow) (F), respectively. Bar, 10 μm.

seed yield. It also had weak growth and was more susceptible to diseases during and after flowering in the humid and high temperature environment, resulting in quick withering. In particular, it showed a barrier of selfing seeds, probably due to self-incompatibility, for its normal anthers were full of pollen grains with high stainability (89.2 ± 4.1%), while the seed set by self-pollination was quite low (1.32 seeds/pod) but very high (9.48 seeds/pod) by bud pollination.

All MAAL plants generally showed the morphology or architecture biased to RBR Oro, but expressed some peculiar features likely associated with each additional C-subgenome chromosome. Attractively, only AAC2 produced the plants with dark green leaves and stems covered with much waxen powder, as Oro did, while the other MAALs gave the plants with slight green leaves and stems. In comparison with RBR Oro, MAAL plants gave rise to decreased vigor, reduced stature and branch number, as well as delayed flowering time except for AAC6 (Figure S2A), probably because of deleterious impact of aneuploidy. Some particular features of RBR Oro were presented by all or some of the MAALs, such as the hindered growth and sensitivity to diseases, barrier of selfing seed sets, as well as procumbent leaves in winter, but AAC2 had a good selfing seed set in higher temperature and AAC9 did not have procumbent leaves in winter. MAALs were distinguishable by their detectable differences in the morphology of leaves from seedlings (Figure 7) and flowers (Figure S2B). Interestingly, six MAALs (except AAC3, AAC4, and AAC9) had high pollen fertility comparable to Oro and RBR Oro (*t* test, $P < 0.05$, Table 4), contrasting the much reduced fertility in the other *B. rapa–B. oleracea* MAALs (Heneen *et al.* 2012).

There existed some detectable differences in morphological characteristics among these MAALs, which are described separately in File S1 and summarized in Table 4.

Discussion

After *B. napus* was pollinated by several crucifers out of the *Brassiceae* tribe, including *Orychophragmus violaceus* (Cheng *et al.* 2002; Hua and Li 2006), *Capsella bursa-pastoris* (Chen *et al.* 2007), *Lesquerella fendleri* (Du *et al.* 2008), *I. indigotica* (Tu *et al.* 2010) and *C. abyssinica* (present study), only nonclassical hybrids were identified and contained the variable chromosome numbers ($2n$ = 19–38) from *B. napus* and few alien chromosomes or DNA sequences. The chromosomes from the pollinators were likely eliminated mostly during certain stages of embryo or hybrid plant development, because of their distant relationships with *B. napus*. The production of *B. napus* euploid plants ($2n$ = 38) might originate from the natural chromosome doubling after the alien elimination, from parthenogenesis of unreduced gametes, or from occasional pollen contamination. Of relevance for the extraction of the A subgenome, the C-subgenome chromosomes were also lost to different extents in individual hybrids, together with those from pollinators that functioned as inducers (Figure 1). Specially, the hybrids with $2n$ = 29 were recurrently obtained in all crosses with these pollinators, and only two representative pairings (1III + 9II + 8I and 10II + 9I) showed that 10 duplicated and 9 individual chromosomes were maintained (Cheng *et al.* 2002; Chen *et al.* 2007; Tu *et al.* 2010; present study). The A-subgenome origin of 10 paired chromosomes and C-subgenome origin of unpaired ones were speculated (Chen *et al.* 2007) but confirmed by the derivation of the restituted *B. rapa* from the hybrids after two more rounds of the induced chromosome eliminations (Tu *et al.* 2010; present study). FISH and molecular analyses of the hybrids ($2n$ = 29) in this study showed the AAC complement with the whole copy of the C subgenome. The completeness of the A subgenome was retained in the progenies of generations and the continuous loss of C-subgenome chromosomes was realized after further induction, finally leading to the production of RBR (Figure 1 and Figure 2).

The introgression of alien DNA elements into the extracted A subgenome probably occurred at a low rate, for such sequences specific for pollinators were detected at low frequencies in the hybrids ($2n$ = 29) with Oro pollinated by *O. violaceus* (Cheng *et al.* 2002) and *I. indigotica* (Tu *et al.*

Table 4 Chromosome pairing, transmission rate of additional chromosomes, and pollen fertility for MAALs

MAALs	Chromosome pairings				Transmission rate ♀ (%)	Transmission rate ♂ (%)	Pollen viability (%)
	Total cells	I^C (%)	III^{AAC} (%)	IV^{AAAA} (%)			
AAC1	119	82 (68.91^{d})	31 (26.05^{a})	6 (5.04^{a})	33.0^{a}	13.3^{a}	93.1 ± 1.3^{B}
AAC2	116	81 (69.83^{cd})	28 (24.14^{a})	7 (6.03^{a})	27.5^{ab}	8.6^{a}	96.2 ± 1.1^{A}
AAC3	108	81 (75.00^{bcd})	24 (22.22^{a})	3 (2.78^{ab})	14.5^{c}	3.2^{b}	80.4 ± 0.6^{D}
AAC4	104	85 (81.73^{ab})	15 (14.42^{b})	4 (3.85^{a})	8.3^{d}	8.8^{a}	71.1 ± 2.3^{E}
AAC5	113	84 (74.34^{bcd})	27 (23.89^{a})	2 (1.77^{ab})	19.4^{bc}	3.3^{b}	86.2 ± 1.8^{C}
AAC6	97	76 (78.35^{bc})	18 (18.56^{ab})	3 (3.09^{ab})	26.3^{ab}	6.7^{ab}	86.0 ± 2.1^{C}
AAC7	103	82 (79.61^{b})	19 (18.45^{ab})	2 (1.94^{ab})	20.5^{bc}	6.0^{ab}	93.4 ± 0.8^{B}
AAC8	123	96 (78.05^{bcd})	26 (21.14^{a})	1 (0.81^{b})	27.4^{ab}	7.5^{a}	89.1 ± 3.1^{BC}
AAC9	101	88 (87.13^{a})	11 (10.89^{b})	2 (1.98^{ab})	7.8^{d}	0.0^{c}	69.0 ± 0.5^{E}
Average	—	76.99	19.97	3.03	20.5	7.2	

Shared letters (a–d) within each association type denote that the values are insignificantly different (χ^2 test, $P < 0.05$). Average of male transmission rate excludes the data from AAC9. Groups A–E detected as significantly different by *t* test, $P < 0.05$.

2010). Subsequently, with the loss of all C-subgenome chromosomes and the alien individual chromosome or segments in the hybrids, the alien introgression would decrease further, *i.e.*, from 3.48% in the hybrid to 1.64% in RBR ZS11 (Table 1). However, the A–C homeologous pairing (1III + 9II + 8I, other than 10II + 9II) in the hybrids ($2n = 29$) could probably move certain segments of the C-subgenome chromosomes to the specific A-subgenome chromosome, as the trivalent occurred in ~60% PMCs (Cheng *et al.* 2002; Tu *et al.* 2010; present study). The exchange rate should be much reduced as more C-subgenome chromosomes were eliminated. Possibly, the transposon activation during the extraction process should cause more genomic changes of RBR (Table 1). Similarly, the preferential loss of C-subgenome chromosomes and different stabilities of three subgenomes (B > A > C) were also observed in the synthesized *Brassica* allohexaploids ($2n = 54$, AABBCC), after being pollinated by *O. violaceus* (Ge *et al.* 2009) or after self-pollinations of successive generations (Zhou *et al.* 2016). The distinct behaviors of these genomes were possibly attributable to their differences in size, inherent cytology, and homeology (Zhou *et al.* 2016).

In another scheme to extract AABB component from allohexaploid bread wheat and restitute the ancestral species, the hybridization between the extant tetraploid wheat ($A^tA^tB^tB^t$, the superscripts denoting the origin of the subgenomes) (*Triticum turgidum* subsp. *durum*) and hexaploid wheat ($A^aA^aB^aB^aD^aD^a$) (*T. aestivum*) was carried out, followed by the nine cycles of backcrossing of the hybrids ($A^{at}A^{at}B^{at}B^{at}D^a$) to the hexaploid wheat to recover the genome ($A^aA^aB^aB^a$) of the restituted wheat (Kerber 1964). The presence of the *Ph1* locus on the 5B chromosome, which prevented homeologous exchanges, likely contributed to the high karyotype stability of the restituted wheat (Zhang *et al.* 2014). Theoretically, the extracted genome should be >99.8% identical to the $A^aA^aB^aB^a$ subgenomes of its bread wheat donor after the ninth backcrossing (Zhang *et al.* 2014). Although the extracted AABB genomes would largely represent those in bread wheat, the minor part of the genomic composition of the extant tetraploid still retained could make them diverge from the original situation to certain extents. So our extraction of the A subgenome from natural *B. napus* by inducing the preferential loss of the C-subgenome chromosomes had advantages for keeping its real constituent, and inversely for *in situ* dissecting of the C subgenome of the same *B. napus* donor. Otherwise, by following the backcrossing scheme of wheat (Kerber 1964), natural *B. napus* ($A^nA^nC^nC^n$) with progenitors unknown was crossed to extant *B. rapa* (A^rA^r) and the resultant hybrid ($A^nA^rC^n$) with the two A subgenomes of different origins was backcrossed as female four times to *B. rapa*, which resulted in the derivation of the diploid AA hybrid containing the lower proportion of the A^n subgenome but much higher proportion of the A^r subgenome than expected, together with some C^n-subgenome introgressions (Pelé *et al.* 2016). The heterogeneity of these two A subgenomes should change the genetic effects of the individual C-subgenome chromosome in established MAALs, which hindered the precise determination of genes and their performance, and specifically the study of genome interplay.

The morphological features in diploid hybrids and derived allotetraploids were of plurality, and ranged from intermediate to parental to transgressive (Soltis *et al.* 2014). The phenotype of RBR should reflect the proximal image of the actual *B. rapa* parent, in consideration of the short history of its hybridization with *B. oleracea* ~7500 yr ago (Chalhoub *et al.* 2014). Although RBR Oro resembled morphologically a winter type of European *B. rapa*, for Oro originated from Europe, it presented obvious genetic divergence from the *B. rapa* types from the Old World or East Asia, as revealed by SSR markers (Guo *et al.* 2014). The causes for the divergence of the A subgenome in *B. napus* were different from those of natural *B. rapa* types, which evolved as independent taxa. As extensively observed, the resynthesized *B. napus* at initial generations showed the changes at genomic, transcriptomic, and proteomic levels (Song *et al.* 1995; Albertin *et al.* 2006; Gaeta *et al.* 2007; Xiong *et al.* 2011; Cui *et al.* 2013). Furthermore, due to the close relationship between A and C subgenomes and the lack of genetic mechanism suppressing the homeologous pairing (Cui *et al.* 2012), high rates of homeologous exchanges involving large segments to single SNPs occurred

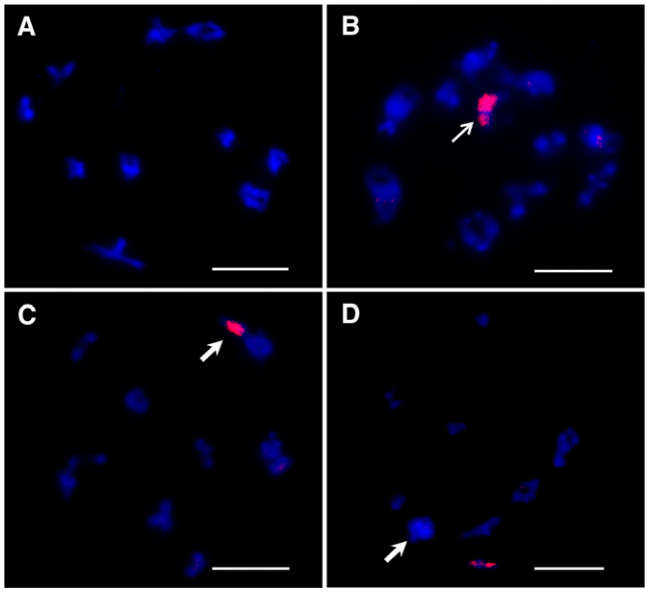

Figure 6 BAC-FISH analyses of meiotic chromosome pairings in MAALs. (A) One PMC at diakinesis of RBR Oro with 10 bivalents of the A subgenome ($10II^{A}$). (B) One diakinesis PMC of AAC1 with $10II^{A}$ and one I^{C} (arrow). (C) One diakinesis PMC of AAC2 with $9II^{A}$ and one allosyndetic trivalent (III^{AAC}; solid arrow). (D) One PMC at diakinesis of AAC2 with $8II^{A}$, one I^{C} (red signal) and one autosyndetic quadrivalent (IV^{AAAA}; big arrow). Bar, 5 μm.

in natural *B. napus* (Chalhoub *et al.* 2014). As *B. napus* cv. Oro was normal, the defects in growth vigor and resistance to biotic and abiotic stresses shown by RBR Oro suggested that some genes of the A subgenome lost their functions, but those of the C subgenome were still active during the evolution. The normal growth and vigor of resynthesized *B. napus* parented by RBR (data not shown) further proved the correction of the altered functions of the AA subgenome by the CC subgenome. Similarly, the extracted tetraploid wheat ($A^{a}A^{a}B^{a}B^{a}$) with a stable karyotype exhibited the wide-ranging phenotypic abnormalities (Kerber 1964; Zhang *et al.* 2014), and many (rather than a few) genes were likely affected in its genome. But the resynthesized allohexaploid wheat parented by extracted tetraploid wheat fully restored normal phenotypes and high fertility, which also demonstrated that the DD subgenome compensated for the compromised functionality of the BBAA subgenomes of bread wheat (Kerber 1964; Zhang *et al.* 2014).

The whole exterior of the synthetic and natural *B. napus* was more biased to the parent *B. oleracea*, particularly by expressing its trait of the deeply green leaves covered with a thicker layer of waxen powder (Cui *et al.* 2012; Heneen *et al.* 2012). So *B. napus* was called *B. oleracea*-type rapeseed in China, and replaced the native *B. rapa* and *B. juncea* for its higher seed yield and stronger resistance to biotic and abiotic stresses. The better resistance of *B. napus* was largely contributed by *B. oleracea*. The weak vigor and strong susceptibility to diseases of RBR also suggested that the functional partitioning of the resistance was enhanced for the C subgenome but attenuated for the A subgenome in *B. napus*. But the biased expression toward only one parental genome (*i.e.*, the genomic asymmetry) at different levels in *B. napus* was not so obvious as in wheat (Feldman *et al.* 2012) and upland cotton (Paterson *et al.* 2012). The characteristic *B. oleracea*-type exterior similarity between AAC2 and Oro indicated that the C2 chromosome harbored the gene(s) for the synthesis of waxen powder. The distinct phenotypes given by the different MAALs suggested the genetic effects of the gene(s) located on the extra C-subgenome chromosomes in the background of the A subgenome (Figure 7 and Figure S2), and some traits not observed in *B. napus* were likely suppressed after genome merger. For example, the trichomes appeared on the leaves of young plants for RBR Oro, but Oro never produces the leaves with the trichomes. As *B. oleracea* did not show this characteristic, the gene(s) from *B. rapa* was silenced in *B. napus*. Only MAAL (AAC7) produced the densely hairy leaves, implying that each C-subgenome chromosome except C7 completely or partly suppressed the expression of this trait.

The cytological diploidization, *i.e.*, the inhibition of pairing to homeologous chromosomes, was fundamental for the evolution success of the allopolyploid species. The cytological process in mostly studied allohexaploid wheat was realized through two independent but complementary systems: the genetic control of pairing (*Ph1* gene) and the physical divergence of the homeologous chromosomes (Feldman and Levy 2012). Owing to the close relatedness between A and C subgenomes in *B. napus*, homeologous pairings and exchanges were frequently detected in synthetic and natural *B. napus* (Nicolas *et al.* 2007; Szadkowski *et al.* 2010, 2011; Cui *et al.* 2012; Chalhoub *et al.* 2014). Specially, whole-chromosome aneuploidy and structural alterations occurred in its progenies of several generations and were biased to those chromosomes with extensive homeology (A1/C1 and A2/C2) (Xiong *et al.* 2011), likely resulting from their nondiploidized meiotic pairing (Cui *et al.* 2012). Accordingly, the two MAALs (AAC1 and AAC2) showed the higher rates of allosyndetic trivalents as well as higher transmission rate of the added C-subgenome chromosomes by both male and female gametes (Table 4). Although diploid-like meiosis in *Brassica* allopolyploids was proposed to be genetically regulated (Prakash 2009), the major QTL *PrBn* (pairing regulator in *B. napus*) found so far to suppress the homeologous pairing functioned only in its haploids (Jenczewski *et al.* 2003). The locus was mapped on linkage group C9, and two minor QTL on C1 and C6 (Liu *et al.* 2006), while its molecular function and mechanism of action remained elusive (Nicolas *et al.* 2009). The lowest frequency of allosyndetic trivalents was observed in our AAC9, potentially owing to the locus. The occurrence of one A-subgenome quadrivalent in all MAALs not in RBR Oro hinted that the extra unpaired C-subgenome chromosomes could promote the crossovers between A-subgenome chromosomes. These data recalled the "interchromosomal effect," whereby crossover rates in inversion heterozygotes were sometimes elevated in collinear parts of the genome (Schultz and Redfield 1951). However, the

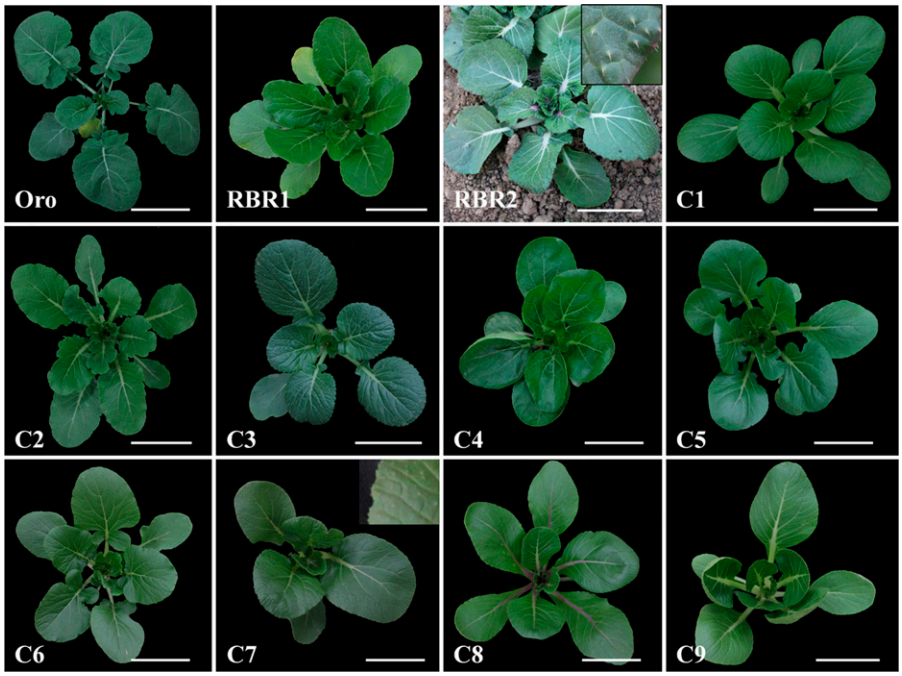

Figure 7 Morphology of donor *B. napus* (Oro), RBR Oro, and MAALs at seedling stage. The seedling and young plant in winter for RBR Oro are given (RBR2). C1–C9 mark nine MAALs. Bar, 15 cm.

corresponding $A^rA^rC^o9$ in MAALs of *B. rapa–B. oleracea* showed the highest frequency of allosyndetic trivalents (Heneen *et al.* 2012), implying that no suppressor-like *PrBn* located on C9 in *B. oleracea*, or no such pairing regulating system was established in newly synthesized *B. napus*, as the *Ph1* gene in wheat also emerged, likely through allopolyploidization (Griffiths *et al.* 2006).

In comparison with the complete set of *B. rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs carrying the individual C-genome chromosomes (Heneen *et al.* 2012), the rates of PMCs with the pairing of 10 bivalents from the A subgenome and the additional univalent of the C subgenome in our MAALs were much higher (68.91–87.13%, averaging 76.99%) than those in *B. rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs (39.0–82.5%, averaging 55.8%). Furthermore, the additional C-subgenome chromosomes in all our MAALs showed only one type of homeologous pairing by forming the trivalent with two A-subgenome chromosomes. But most of C-genome chromosomes in other MAALs had more than one type of homeological pairing partner among the A-genome chromosomes, resulting in the formation of heteromorphic bivalents, trivalents, or pentavalents (Heneen *et al.* 2012). The less flexibility of homeologous pairing in our MAALs also showed that not only the A and C subgenomes of natural *B. napus*, which coevolved in the same nuclei for thousands of years, underwent more physical divergence from each other than from those of extant diploids, but also the homeologous pairing was weakened or restricted probably by some genetic control, which should develop during the evolution.

As *Brassica* genomes experienced a *Brassiceae* lineage-specific whole genome triplication, which was followed by abundant rearrangements and divergence (Lysak *et al.* 2005; Wang *et al.* 2011b; Liu *et al.* 2014), the A and C subgenomes in *B. napus* would be complex mosaics of triplicated ancestral genomic blocks. Therefore, the scaffold assignment to the correct chromosomes should be a challenge for the genome sequencing of *B. napus*, because ~28% assembly scaffolds in *B. oleracea* (Liu *et al.* 2014) and ~13% in *B. napus* were undetermined (Chalhoub *et al.* 2014). With this complete set of MAALs with each C-genome chromosome isolated, PCR amplification of specific gene markers from the C genome across MAALs was feasible for anchoring those nonanchored C-subgenome scaffolds to corresponding chromosomes. In total, 22 comparatively large scaffolds were successfully anchored to their specific C-subgenome chromosomes. However, it should be pointed out that the method of PCR amplification was laborious and time consuming, because of the huge number of undetermined scaffolds and small size for the majority of them. With aneuploidy lines, two studies employing chromosome-based sequencing were performed to decipher the genome information of allohexaploid wheat (Mayer *et al.* 2014), which highlighted the possibility of drawing support from the combination of current MAALs and high-throughput sequencing, such as resequencing as well as RNA-sequencing technology, to overcome barriers in genome sequencing of *B. napus*.

In conclusion, the distinct stabilities of two subgenomes in young allotetraploid *B. napus* made it feasible to first extract the more stable A subgenome and restitute the progenitor *B. rapa*, by inducing the biased loss of the unstable C subgenome. Subsequently, the C subgenome of the same *B. napus* genotype was readily dissected with each chromosome added to the extracted A subgenome. The RBR and the whole set of additions provided direct observation of the phenotypes of the progenitors after the evolution of thousands of

years, and also provided unprecedent insights into structure and function of two subgenomes and their interplay in this allotetraploid. The ongoing studies of these novel materials for genome, transcriptome, DNA methylation, and proteome are expected to reveal more information about the contributions and interactions of two progenitors that produced *B. napus*.

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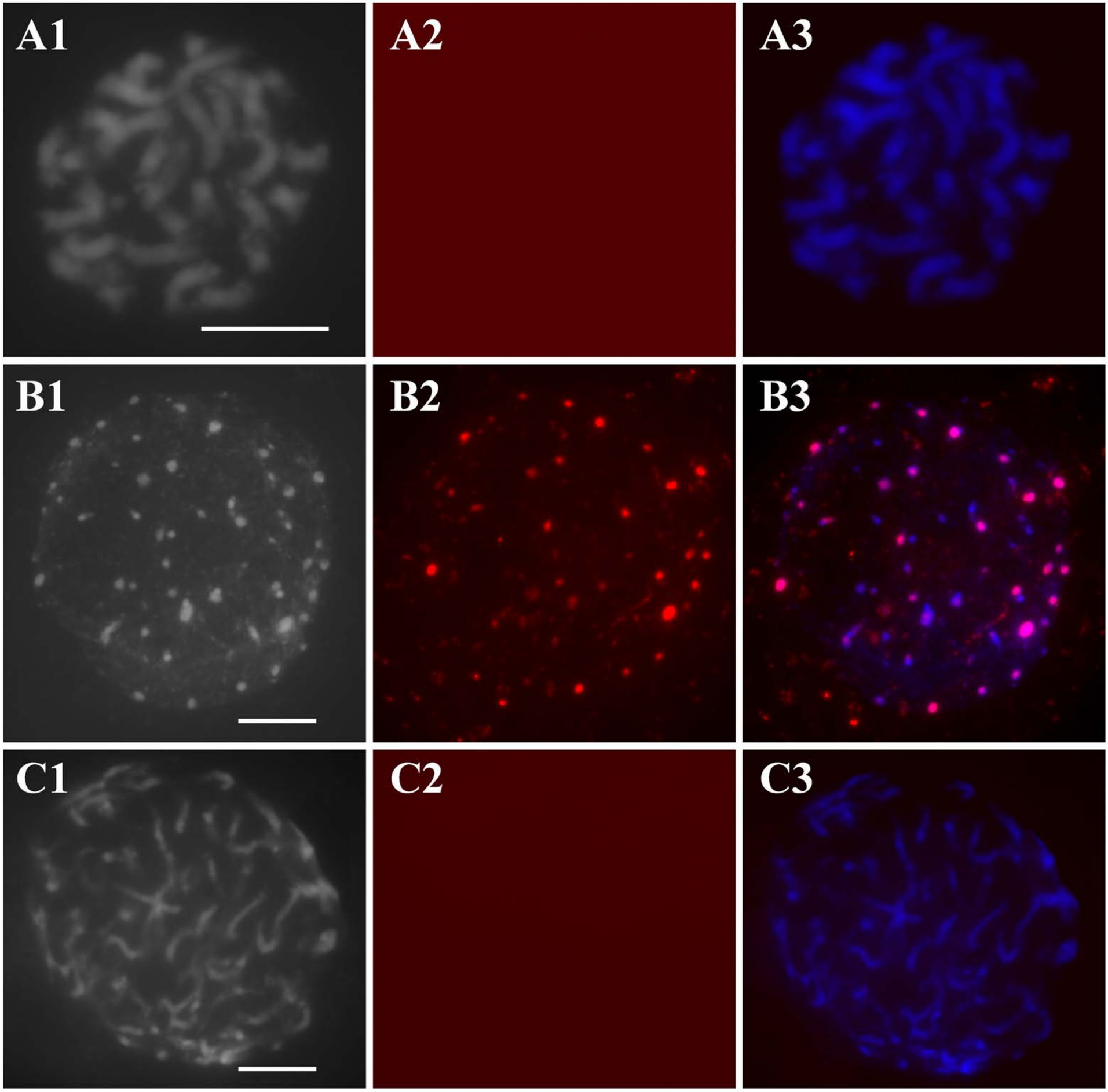
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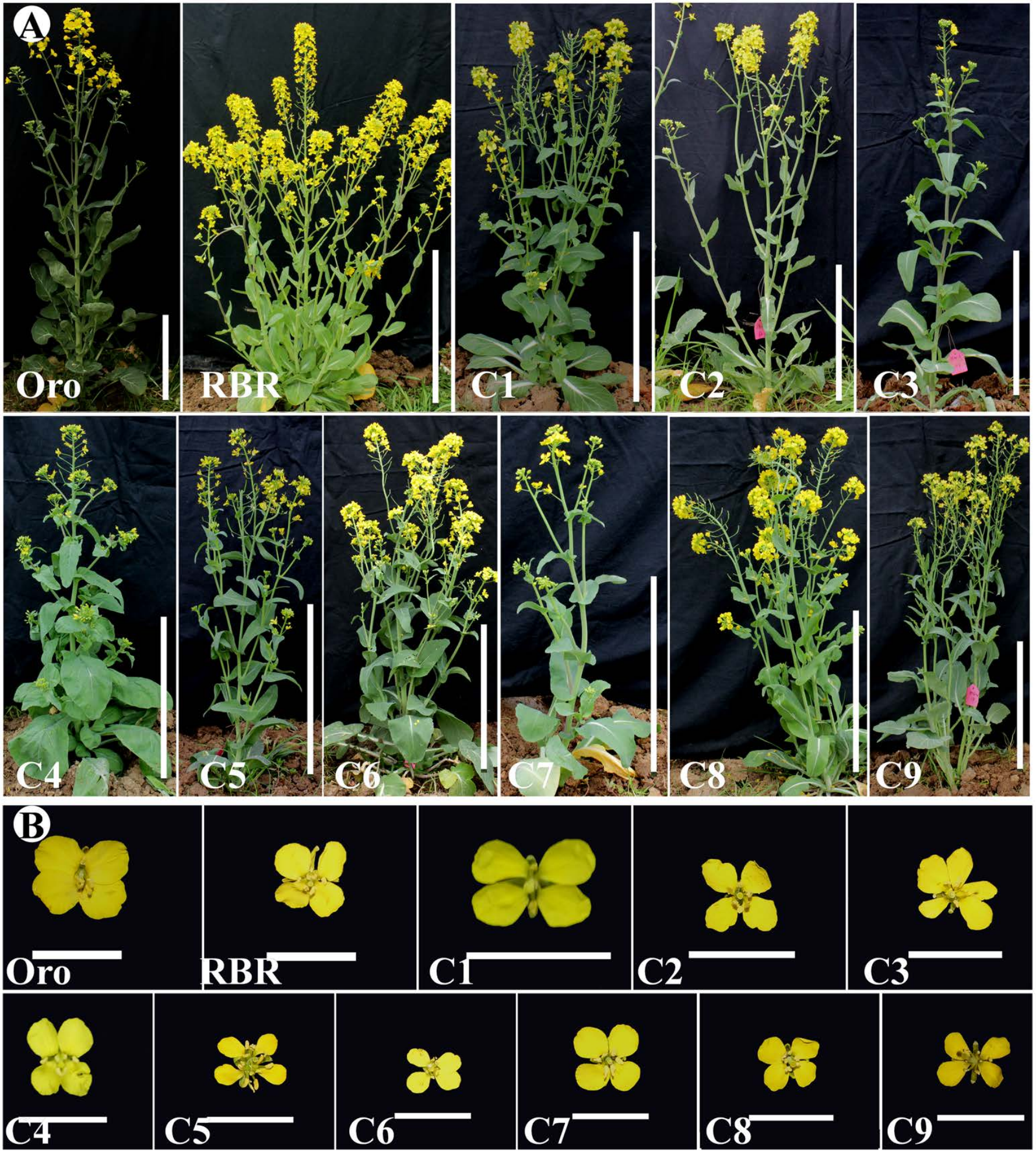
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C3
C4
C5
C6
C7
C8
C9

Table S1 The list of C chromosome specific gene primers for identification of a complete set of MAALs

Primer number	Gene ID	Gene locus	Target Chromosome*	Forward sequence	Reverse sequence	Size of product (bp)	Tm value(℃)
C1c	BnaC01g20520D	14330034 -14330509	C1a	CTCCAGATATTCAGCAATTCG	TAGAGAGAGAGGATCAACGGC	324	55
C1o	BnaC01g31840D	30862940 - 30864210	C1b	AAGGTATGCGGATCTCTCAAGT	CATAGCTCAAGACGTCACGGAA	322	57
C1p	BnaC01g32060D	31174209 - 31176556	C1b	TTTCATGTTTCACCGACTGTTT	CTGCCATTGATCGAGATAGAAA	336	55
C1v	BnaC01g29450D	27805790 -27807456	C1b	CTGAATAATCTCTCTGTCTATAGGT	TGAACCAACTCTAACTCTATTCCTA	246	57
C2b	BnaC02g07370D	3980643 -3983556	C2a	CCAACGAACGAGGCAAGTG	TTTCGAGGCACATCAAGCA	405	57
C2c	BnaC02g07560D	4145454-4150528	C2a	CTAGAGATTAACCTACACGTCC	CAGTTAGACATGGCATTCAA	242	57
C2e	BnaC02g45250D	undetermined	C2_random	TGACGCAATTTTTATTTCCGACGA	ACCCTTAGATCGATTGATCTTGT	484	55
C2g	BnaC02g38500D	41469818 - 41472510	C2b	TTGGAAGTCGGTGAGTATC	TTGACCCGAGAAAGAAGAT	449	55
C2h	BnaC02g40690D	43735079 - 43737186	C2b	AACAGCAGAGGAAACCAATCT	ACCACCTCTAACTACTCGAAC	391	55
C3e	BnaC03g18470D	9470365 - 9473709	C3a	TATAATCGTACAAGTCGGCAC	GCCTGAAACACACAAGGATTT	314	55
C3g	BnaC03g70340D	59989920 - 59991885	C3b	GTGGCACTTATTCAACAACA	GCTCGATCAAACACTACTGC	282	57
C3h	BnaC03g69660D	59443098 - 59445645	C3b	AAACCTATTCGTGTTCGTTCTC	TTCATTCTAACTACCGGCAAAT	317	55
C3i	BnaC03g16130D	8149599 - 8151810	C3a	AAATGCCACCAACAGCTTCCTT	TGAACTGGAGAGTACCTTCTTGA	265	55
C4b	BnaC04g11470D	8978893 - 8982129	C4a	ATCTTCTCCTTAACGCGTTT	CACCAAGAAGACAACACCATATA	398	55
C4c	BnaC04g44230D	44354526 - 44355241	C4b	CATGTGTGAGAGAGAGATAGTG	TAAAGACCAGAGATCCGAACAA	335	57
C4e	BnaC04g12960D	10255228 - 10256676	C4a	ACAAACATTTCCTGATCTTCCT	GTCGATTCCGGTTTCTCTAAGT	388	55
C4f	BnaC04g15230D	12961369 - 12962575	C4a	GACGCGTATATAGTGCATCAG	ATCCACAGCTATTCTCACTCT	301	55
C4h	BnaC04g25120D	26067044 - 26071423	C4b	TGGAACCTCATCGCTTATT	GTTGTGCTTATTCTATTCGTT	341	53
C4i	BnaC04g25250D	26194282 -26195533	C4a	TCAAAGAAGAAGGTCCATGCA	CGTGTATCTTTCAGGGTAGGA	487	57
C4j	BnaC04g11750D	9211121 - 9215359	C4a	TGGAGTTTCCGTAGTTTATGTGTGTT	ACAAATCCAGAGAGTCCATTATATCGA	429	55
C5a	BnaC05g26960D	23389162 - 23390341	C5b	GAAGCGAGGAGTAGAGAAGAC	TTTTACAGGTGAAGGAACAGC	362	57
C5e	BnaC05g00200D	73985 - 74636	C5a	GACATGCAGTTCGATCCTACT	GGATGACATCTTTCAAGCCC	417	57
C5g	BnaC05g26290D	22308267 - 22308524	C5a	ACGAACCGAGCAGATTACGTAG	CAATGATGCCCAACCCCTG	246	57
C5h	BnaC05g27990D	25548819 - 25550396	C5b	AGCGCCTGAGATTACAAACTG	AATCCCATTATCCAAGGTTCC	356	55

Table S1 The list of C chromosome specific gene primers for identification of a complete set of MAALs (continue)

C5i	BnaC05g02520D	1230796 - 1234120	C5a	ATCTACATAAAGGAACTACATCCCAT	TCGCCTACAGGCTCTAAAAAATCT	764	51
C6b	BnaC06g08180D	9078388 - 9079465	C6a	CCTTGCAGCTTCAGATATCTC	TGGAGCACATCAAAATTAGCA	323	55
C6e	BnaC06g36250D	34842800 - 34845278	C6b	ACCACCACAACTTTTACCAG	TGAGAAGGAGTTTCGTAAAGGG	554	57
C6h	BnaC06g11200D	13156807 - 13156995	C6a	ATGAACCTTGACTTATTTTGCCA	TGGCTCTGTTCCCTCTCTA	321	57
C6k	BnaC06g25500D	27150009 - 27154346	C6b	AGGTAGATCTACCCTAAGATTGTATT	AAAGTCATACATTCTAATCGACGTG	757	55
C7c	BnaC07g46220D	43945051 - 43945517	C7b	TTGTTGACCATCGTTTCTCTCT	CTTTGCAGTTAGCTTTTCCAAT	379	55
C7d	BnaC07g30320D	34859446 -34861960	C7b	ATACATTCAGAAGGAGCGCT	CGCCGTAGTTAGATGTGACA	475	57
C7e	BnaC07g34040D	36982832 - 36984967	C7b	CCACCTGAGGATATTGCTACT	AGAGTAATAGCTGGGACTTGT	293	57
C7f	BnaC07g34310D	37142657 - 37144100	C7b	CTTTCTGATCCACTTGATACTG	TAGACATGCCAAGAAGTTGAT	382	55
C7z	BnaC07g28980D	33928154 - 33930739	C7b	AGTTTCATTACAATCAATATTGAACTTGA	AAGTATATTGAAGCAAAGCTGCAGA	444	51
C7i	BnaC07g00330D	605923 - 607377	C7a	TAGTAGTGAGTGAGCGAGGGA	CAGACCAGGTGTTGATGAAAT	219	55
C7m	BnaC07g11290D	16972881 - 16974006	C7a	TTGCAGTCAGATTCAAGAACTTC	AGATACTCCTGTCGGCTACTA	482	57
C8a	BnaC08g06960D	9684080 - 9685841	C8a	TCATCAGACCGATTTGGAACT	TTCCTGCCCAAATAACATCAA	274	55
C8b	BnaC08g27250D	28323933 - 28324907	C8b	TGAAGGTATAAACACTGGATTG	AGGCAGGAGACATGAACATA	217	55
C8f	BnaC08g14450D	19214692 - 19216202	C8a	AGGAGCTTGTCACGCCTAG	TACGTTGGGATTGGCTGAA	410	57
C8g	BnaC08g48020D	2986896 - 2988732	C8b	GATTACGAAGATGAAGAAGCG	TGTTCTTGTACACACCCAG	271	55
C8h	BnaC08g20810D	23416000 - 23421543	C8b	CTCTGGATTTAAATGTACGATG	AGTCTTATCCAACCAAGTATAT	358	55
C8i	BnaC08g36680D	33815790 - 33819038	C8b	TCTCAGACGCAGCAGGAGCA	ACTCCCTCACATTGATAGTAGCCT	422	55
C9a	BnaC09g40030D	42562268 - 42567591	C9b	TCCATGTTCCTCTGATGCTA	AGCTGAACGAATGACAGTTGG	335	57
C9e	BnaC09g28260D	30570207 - 30570842	C9b	ATGATGATGAAGGGTTTGCA	CATTTTATCCCACTAGATGCGT	213	55
C9f	BnaC09g52810D	undetermined	C9_random	GTGGATCTCAAGCTTCTCTAC	CCACCCATATAGAGCAATGAG	393	57
C9g	BnaC09g40440D	42878002 - 42879865	C9b	GCTCCTCGTCCTTATATTGCT	TCTTCTACGGTTTTCCTCTTTG	445	57
C9i	BnaC09g27290D	29041826 - 29043953	C9b	TCCACGAGGGAGAATAAACCAT	ACAATCGCTCCACCTTATCCGA	376	55
C9w	BnaC09g12210D	8644000 - 8644369	C9a	AGCTCTCTTCCATGTAATCTCA	TCAAATAGGCGAAGAGTACAGA	314	55
C9x	BnaC09g12400D	8801885 - 8802247	C9a	CATTGTTTATTGTTCTGGTCGCA	TTCGCAGTCGCATAGGAAATT	335	55

1 * The letter *a* represented for the gene located on the first half of one assembled chromosome of B. napus genome sequencing, *b* for the
2 latter part of the chromosome and *r* for an uncertain location on the chromosome

Table S2 Summary of transmission rates by female and male gamete for MAALs

	Transmission rate by female gamete ♀			Transmission rate by male gamete ♂		
	Total plants*	Target plants	Transmission rates (%)	Total plants	Target plants	Transmission rates (%)
AAC1	48/52	15/18	33.00	75	10	13.33
AAC2	50/52	12/16	27.45	93	8	8.60
AAC3	42/89	5/14	14.50	93	3	3.23
AAC4	30/90	4/6	8.33	91	8	8.79
AAC5	43/60	9/11	19.42	61	2	3.28
AAC6	15/80	4/21	26.32	75	5	6.67
AAC7	47/80	10/16	20.47	84	5	5.95
AAC8	48/69	11/21	27.35	93	7	7.53
AAC9	116	9	7.76	66	0	0.00

*Two plants for MAAL per chromosome were used to assess female transmission rates except AAC9..

Table S3 List of selected scaffolds belonging to non-anchored Cn-subgenome determined by amplification of specific markers in MAALs

Scaffolds		Anchoring gene	Primers	Forward sequence	Reverse sequence	Target chromosome
ID	Size(Mb)					
scaffold_136	1.206	BnaCnng00090D	Cn11	CACTGTGTTTCTGAGTATCCAT	CCAATAACTCCTGCACAATAAC	C7
		BnaCnng00120D	Cn13	GATATATGTTTTGACAACAATGTGT	CTAATGTTAAAACCTCACAGCT	
		BnaCnng00140D	Cn15	TGGAAGTGGACGAAGAGGTGA	TCACAACCACCACCTCCTTCG	
		BnaCnng00170D	Cn17	TCACTTATCTGTTTGTTTCTCA	ATTCGTCTTTTTCTTTGTTTGT	
		BnaCnng00040D	Cn6	GTCGAGTTTCCATTTCTTACCTA	GAGACTGTGAATCCGAGTAATAT	
		BnaCnng00060D	Cn8	AAAATGGGTGACGACGGTGTGAA	TTCCACTCTCTCAGCAGCATAAG	
scaffold_189	1.001	BnaCnng01970D	Cn32	AAGAAGAAGGCAAGAAGCTGT	GTTTCTCAGAGCTTTGTTGGTCT	C3
		BnaCnng02050D	Cn33	GTCTCTTTGATGTTTCTGCTTGGA	GATTCACCAAGGGAAAGAAGAA	
		BnaCnng02040D	Cn34	TCCATACCTAAAAATATATGTTCCGT	CTTTTCTTCTGGTCCAACTTCA	
scaffold_1961	0.049	BnaCnng34630D	Cn2	CCAGTTAAGAGTTTCACCTTTG	CAACACCAGGACTAGGACC	C8
scaffold_217	0.916	BnaCnng02720D	Cn23	CCAAAAACACAAGAGTACCCA	CACAGCGTAATTACAGACACT	C9
		BnaCnng02700D	Cn24	CGAAGATCCCTCCTGTAAGAT	TCAAGAGCATCTGAGACCTTT	
scaffold_264	0.806	BnaCnng04300D	Cn26	AGTAGGAGTACGCATTTGTTT	CGTTGAACTTGCCTCGAAACA	C4
		BnaCnng04400D	Cn28	ACTGTAGCGTCTGAGTTTTGA	AATTACTGTACCTGTTCGTCGAT	
		BnaCnng04060D	Cn29	GGATTAGGGTTTATTTTTCGTGAT	TGCAATTGAAGTGTAGGATTCTGA	
scaffold_372	0.614	BnaCnng04520D	Cn35	ATGGCAGGAAGTATCTCTCTAA	TAAAGTCTCCTCCTACTACCCA	C3
		BnaCnng04800D	Cn37	TTGGTCAACTCTTCGGTGTATAT	GCTTCTTAGATGTCGTTTGAGTT	
		BnaCnng04700D	Cn38	CCCTGAAGCATTGCATAACTA	CTAGAAAATCAGCAACAACGT	
scaffold_426	0.551	BnaCnng04980D	Cn39	AATAAACGAACAGTGTCAGCTT	AAACAACAAATCAAGCAAGTCA	C8
scaffold_439	0.535	BnaCnng06150D	Cn46	CGATAAGCCCTAATGATCTGGA	GTAACGAAACCGATAAGCTCAT	C5
		BnaCnng06340D	Cn45	ATGACCAAACGTGATTTAGAACT	TTACCCATGGCAGCTTAAAAT	
scaffold_502	0.462	BnaCnng06980D	Cn49	TTTCAGTAGTTTGTTTCCTCCT	AGTATTTCAATCTCGGTGCA	C8
scaffold_521	0.44	BnaCnng07200D	Cn54	CGTGAATGAAGAAGAGAACAGAA	GACGGTAAGACAAAGTGATAGTT	C5
		BnaCnng07230D	Cn53	GCATGAATCAATGGGAACTCAA	CCGCTAAATCAGTTGGTATCAGA	

Table S3 List of selected scaffolds belonging to non-anchored Cn-subgenome determined by amplification of specific markers in MAALs (coutinue)

scaffold_536	0.432	BnaCnng07290D	Cn56	GCTAGTGGTCTTGAGGTAGTTA	ATCCATCTCAAAAACCAACTCA	C6
		BnaCnng07540D	Cn57	AAGACAACAACAGACGGTACT	AAACCTTCCTTCGAGAAACCT	
scaffold_595	0.369	BnaCnng07910D	Cn62	CTTATCACAAACAGACACTCCAA	ACCGCAACGAAATTCCTTAT	C6
		BnaCnng07970D	Cn61	CCAGATTCGTTGCTTTGTCA	TTTCCACAACATGATCGTCT	
scaffold_610	0.354	BnaCnng08000D	Cn63	CTCTCCTCTTCATCGACCTT	AAAAAAGAAGCACGCGATGAT	C2
		BnaCnng08020D	Cn64	CTGGTAACATCAAGGTTGGTA	TGTTTCATAGAATTCAAATGTGGTT	
		BnaCnng08070D	Cn65	ATGGCCAGAATCATCATCAGA	TTCTACCAAAGCGGAAGCATA	
scaffold_618	0.347	BnaCnng08340D	Cn67	ATTTGGAGAGATCATTGGAAGA	ATGATGAAGGAGGCAAATACTA	C2
		BnaCnng08400D	Cn68	TCAGAAAATCCATGTCAGCAAT	TGATTGTATTGGTACGTTGCTT	
scaffold_628	0.341	BnaCnng08950D	Cn1	TGTCCTTGAAGAGTCGCTTTG	CTTCACCAATGCATAGTGTCA	C8
scaffold_666	0.318	BnaCnng09260D	Cn71	CTCACTCTCCGATCAGGTAA	TCACCACAACAACACTATACAT	C8
scaffold_697	0.298	BnaCnng09640D	Cn78	CTGATGCTCCTTCAACTAACTGA	ATTGATTTCTACCGCATTACAGA	C2
		BnaCnng09880D	Cn77	ATGTGGAAGCTTTTGGAACA	CATTCACTATCACTTCAAGCCT	
scaffold_700	0.297	BnaCnng09960D	Cn80	AAGTGGAATATGCTCATGCCT	GTGACGTGTGATAGTAATGGGT	C9
		BnaCnng10030D	Cn120	CGCGTTCATTACAATATCCACA	GATGCAAATATAAGGTTCGCTGA	
		BnaCnng10150D	Cn79	CCCAACGGAAGAGATGTTAAA	GAACCGTCAACACAATCATTA	
scaffold_761	0.262	BnaCnng11020D	Cn90	TGTTCGTATTTTCAACTCTTACTGA	TGCCTAAAGGTCACAGATTAACA	C5
		BnaCnng11030D	Cn89	CGAAAGGTTGCCGAGAAAGA	TTTGGGTTGGTATGGTATCTCA	
scaffold_804	0.237	BnaCnng11210D	Cn92	ATGGCTTCTTCTTCTAGTGTAAA	CATAGGTTCCCAAATTGTTCT	C4
scaffold_807	0.235	BnaCnng11380D	Cn105	GATTGACGTTCTTGACTAACAGA	ACAAACAAATCCTAAGAAAATCGA	C2
scaffold_810	0.231	BnaCnng11560D	Cn106	CAGCAGAGAGGAGTCATTTAT	TCCATTGACTAGAGCTCTGT	C4

Typical characteristics for each of MAALs

AAC1 plants had oval, glabrous, and convex leaves with clear veins but no lobes, and produced flowers with two long and two short stamens under the low temperature of the initial flowering stage, but the normal flowers with six stamens under the higher temperature (>15°C) of the later stage. AAC2 plants presented long and slender, sparsely hairy and dark green leaves with 2-3 minute lobes, as well as waxen powder similar to the leaves of Oro. The majority of AAC2 plants showed normal flowers and dramatically the highest pollen viability up to the rate of 96.2% (t-test, $p<0.05$), and the minority had variant flowers with 2-4 or no stamens. Intriguingly, AAC2 plants had the best seed-set by self-pollination and highest thousand-seed weight (2.86±0.11g, t-test, $p<0.05$) among MAALs. AAC3 plants gave glabrous, dark green and the smallest orbiculate leaves with one small lobe. They showed severely decreased vigour, the fewest branches as well as delayed flowering time about 10 days later than OroA. Their flowers had mainly five petals and 4-6 stamens. AAC4 plants displayed compact and orbiculate leaves with and one small lobe, easily distinguishable from other MAALs. They grew slowly at the early stage but had large architecture after bolting. AAC5 plants had glabrous and deltoid leaves with one small lobe and sparse hairs on margin. Their deformed flowers had 6-10 stamens and 2-3 pistils in some cases, but only one could ultimately developed into pod. AAC6 plants gave slightly green leaves with two obvious lobes similar to Oro. When grown in October in Wuhan, they flowered before the winter came without vernalization requirement, about two months earlier than OroA, while the remainder MAALs flowered in next spring. AAC7 plants at seedling stage produced the leaves covered with dense hairs, an obvious marker for identification. They had smallest plant

architecture and showed the second latest flowering time, about seven days later than OroA. AAC8 plants had glabrous and rhombic leaves, and gave the smallest seeds (1.29±0.10g, t-test, $p<0.05$) among these MAALs. AAC9 plants expressed a compact plant type with glabrous and orbiculate leaves, and the leaves kept erect while those of other lines crawled over the ground in cold winter. They produced the smallest flowers with the lowest pollen viability (69.0±0.5%, t-test, $p<0.05$).