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Karyotype and Identification of All Homoeologous Chromosomes of Allopolyploid *Brassica napus* and Its Diploid Progenitors

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ABSTRACT

Investigating recombination of homoeologous chromosomes in allopolyploid species is central to understanding plant breeding and evolution. However, examining chromosome pairing in the allotetraploid *Brassica napus* has been hampered by the lack of chromosome-specific molecular probes. In this study, we establish the identification of all homoeologous chromosomes of allopolyploid *B. napus* by using robust molecular cytogenetic karyotypes developed for the progenitor species *Brassica rapa* (A genome) and *Brassica oleracea* (C genome). The identification of every chromosome among these three Brassica species utilized genetically mapped bacterial artificial chromosomes (BACs) from *B. rapa* as probes for fluorescent *in situ* hybridization (FISH). With this BAC-FISH data, a second karyotype was developed using two BACs that contained repetitive DNA sequences and the ubiquitous ribosomal and pericentromere repeats. Using this diagnostic probe mix and a BAC that contained a C-genome repeat in two successive hybridizations allowed for routine identification of the corresponding homoeologous chromosomes between the A and C genomes of *B. napus*. When applied to the *B. napus* cultivar Stellar, we detected one chromosomal rearrangement relative to the parental karyotypes. This robust novel chromosomal painting technique will have biological applications for the understanding of chromosome pairing, homoeologous recombination, and genome evolution in the genus Brassica and will facilitate new applied breeding technologies that rely upon identification of chromosomes.

ALTHOUGH whole-genome duplications (polyploidy) occur in animal and plant lineages, it is generally tolerated to a greater degree in plants, fish, and frogs compared to mammals and birds (Mable 2004; Comai 2005; Otto 2007). Up to 80% of flowering plant species have been estimated to have undergone recent polyploid events in their ancestry (Masterson 1994; Ramsey and Schemske 1998, 2002; Otto and Whitton 2000; Wood *et al.* 2009). However, numerous ancient polyploidy events have been indentified in genomic studies, so at a deep level all angiosperms may have a polyploidy history (reviewed in Soltis *et al.* 2009; Van De Peer *et al.* 2009). Compared to their progenitors, polyploids may display novel morphological and physiological traits that may contribute to speciation (Ramsey and Schemske 2002; Rieseberg and Willis 2007; Leitch and Leitch 2008; Soltis and Soltis 2009), with 15% of angiosperm and 31% of fern speciation events accompanied by polyploidy (Wood *et al.* 2009). Polyploidy has been studied in crops, including wheat, oat, sugarcane, soybean, banana, potato, coffee, tobacco, and cotton (Stebbins 1950, 1971; Leitch and Bennett 1997; Matzke *et al.* 1999; Osborn *et al.* 2003a,b; Adams and Wendel 2005; Chen 2007; Dubcovsky and Dvorak 2007). *Brassica napus* (also known as canola, oilseed rape, Swede) has been an important model for studying the genome constitution of allopolyploids (reviewed by Cifuentes *et al.* 2010; Gaeta and Pires 2010; Pires and Gaeta 2010).

The genus Brassica contains a number of diploid and allopolyploid species including important vegetable, condiment, and oilseed crops. Six of these agriculturally important species can be classified into three basic diploid cytodemes (A, B, and C; n = 10, 8, and 9, respectively) and their allopolyploid hybrids (AB, AC, and BC) as demonstrated in a classical cytogenetic study by U, Nagahara (1935). *B. napus* (AACC; $2n = 38$) is an allopolyploid species formed by the hybridization of ancestors of *Brassica rapa* (AA; $2n = 20$) and *Brassica oleracea* (CC, $2n = 18$), while allopolyploid *Brassica carinata* (BBCC, $2n = 34$) is formed from *Brassica nigra* (BB, $2n = 16$) and *B. oleracea*, and allopolyploid *Brassica juncea* (AABB, $2n = 36$) is formed from *B. rapa* and *B. nigra* (U, Nagahara 1935). Judging from sequence variations within chromosome segments, domesticated *B. napus* has A- and C-genome components that have had few genetic changes relative to the presumed progenitor lines of *B. rapa* and *B. oleracea* (Rana *et al.* 2004; Cheung *et al.* 2009). Genetic mapping studies of

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B. napus cultivars have revealed only a few chromosomal rearrangements caused by recombination between homoeologous regions of the A and C genomes (Parkin *et al.* 1995; Sharpe *et al.* 1995; Jenczewski *et al.* 2003; Osborn *et al.* 2003a; Udall *et al.* 2005). In contrast, studies in resynthesized *B. napus* show that the time period immediately subsequent to allopolyploidization can be tumultuous and dynamic, involving extensive chromosomal rearrangements (Song *et al.* 1995; Pires *et al.* 2004; Lukens *et al.* 2006; Gaeta *et al.* 2007; Cifuentes *et al.* 2010; Gaeta and Pires 2010; Szadkowski *et al.* 2010).

Brassica species have been the subject of extensive molecular cytogenetic analyses during the past decade; however, development of karotypes for Brassica has been challenging due to their small chromosome size and the lack of distinct karyological features of metaphase chromosomes (Fukui *et al.* 1998; Snowdon 2007). Several karyotypes have been published for Brassica species on the basis of different staining methods, such as Giemsa staining, C-banding, CMA3/4′-6-diamidino-2-phenylindole (DAPI) fluorescent staining, silver staining, and fluorescence *in situ* hybridization (FISH) with rDNA or pericentromeric tandem repeats (Olin-Faith and Hennen 1992; Maluszynska and Heslop-Harrison 1993; Cheng *et al.* 1995; Snowdon *et al.* 1997; Armstrong *et al.* 1998; Fukui *et al.* 1998; Hasterok *et al.* 2001, 2005, 2006; Howell *et al.* 2002; Kulak *et al.* 2002; Snowdon *et al.* 2002; Ziolkowski and Sadowski 2002; Koo *et al.* 2004; Maluszynska and Hasterok 2005; Lim *et al.* 2005, 2007). However, given the lack of specific chromosomal markers, even the best karyotypes developed could distinguish only six chromosomes in diploid *B. rapa* and three chromosomes in diploid *B. oleracea*. With four chromosomes in *B. rapa* and six chromosomes in *B. oleracea* lacking distinct karyological features, the identification of all the chromosomes in allopolyploid *B. napus* was impossible. Adding to the confusion, different researchers used separate systems of chromosome nomenclature. Finally, the use of genomic *in situ* hybridization (GISH) to distinguish the progenitor diploid A and C progenitor genomes failed in allopolyploid *B. napus* (Snowdon *et al.* 1997), although GISH has allowed the visualization of the B diploid genome in allopolyploid *B. juncea* (Snowdon *et al.* 1997; Maluszynska and Hasterok 2005) and in ABC trigenomic Brassica hybrids (Ge and Li 2007). GISH has also distinguished Brassica chromosomes from separate species in intergeneric hybrids of Brassica crossed to Lesquerella, Orychrophragmus, Raphanus, and Isatis (Sharzhinskaya *et al.* 1998; Hua *et al.* 2006; Liu and Li 2007; Du *et al.* 2008; Tu *et al.* 2008).

Recently, two developments have improved Brassica molecular cytogenetic approaches. The first improvement is the application of chromosome-specific bacterial artificial chromosome (BAC) probes and new repetitive sequences, which has facilitated chromosome identification in *B. rapa* and *B. oleracea* (Howell *et al.* 2002, 2008; Koo *et al.* 2004; Lim *et al.* 2005, 2007; Mun *et al.* 2008; Feng *et al.* 2009; Kim *et al.* 2009). Howell *et al.* (2002) made a first step toward integration of all nine linkage groups of the *B. oleracea* genetic map to the corresponding chromosomes using BACs as probes for FISH hybridization. Similarly, the genetic linkage map and the *B. rapa* cytogenetic map was integrated by BAC-FISH (Mun *et al.* 2008; Kim *et al.* 2009). The second improvement in *B. napus* cytogenetics is the ability to identify the C genome either by using a BAC clone containing a ubiquitous C-genome repeat (Alix *et al.* 2008) or by using a modified GISH technique that uses a repetitive probe for blocking DNA (Howell *et al.* 2008). The latter study not only identified the A and C genomes in *B. napus*, but also used a sequential procedure with FISH and GISH that allowed for direct visualization of a known reciprocal translocation (Howell *et al.* 2005, 2008). Despite these advances, to date there is no complete *B. napus* molecular cytogenetic karyotype that identifies all of the A- and C-genome homoeologous chromosomes.

In this study, we establish the identification of all homoeologous chromosomes of allopolyploid *B. napus* from karyotypes developed from the diploid progenitor species *B. rapa* and *B. oleracea*. First, we identified every chromosome among these three Brassica species by using genetically mapped *B. rapa* BACs as FISH probes. We then developed a second karyotype probe mixture using two *B. rapa* BAC clones containing repetitive DNA sequences together with 45S rDNA, 5S rDNA, and two 176-bp pericentromere satellite repeats (CentBr1 and CentBr2). Using this diagnostic karyotype probe mix and a BAC that contained a C-genome repeat in two successive hybridizations allowed for routine identification of all the chromosomes of *B. rapa*, *B. oleracea*, and *B. napus*. Here we report the details of this novel chromosomal painting technique and the initial observations of a *B. napus* cultivar and discuss how this new karyotype tool kit will facilitate the understanding of chromosome pairing, homoeologous recombination, and genome evolution in the genus Brassica.

MATERIALS AND METHODS

Plant materials: *B. rapa* doubled haploid line IMB218, *B. rapa* ssp. *pekinensis* inbred line Chiifu 401, *B. oleracea* doubled haploid line TO1000, resynthesized *B. napus* line EL3000-S0, and *B. napus* Stellar were used for cytological studies.

Selection of chromosome-specific BAC-FISH probes: The BACs of *B. rapa* used in this study came from a BAC library developed from the Chiifu 401 genotype (Park *et al.* 2005). The BACs were located on a high-density *B. rapa* linkage map using a combination of sequence-based genetic mapping and fingerprint contig data and FISH (Kim *et al.* 2006, 2009; Mun *et al.* 2008). To increase the likelihood that the two chromosome-specific BACs from each chromosome would be

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hybridized to both ends of chromosomal arms, markers that are located at the ends of each linkage group were used to select the BACs. All of the 20 selected chromosome-specific BACs consistently produced strong and unambiguous FISH signals. Table 1 summarizes the genetic and cytological locations of the 20 BACs. All 20 BACs with other repeated sequences also were localized on mitotic chromosomes of *B. oleracea* and *B. napus*. To confirm the location of the two BACs on each chromosome in *B. rapa* in the context of repetitive elements, we conducted independent hybridization experiments for each pair of BACs. The complementarily labeled BAC pairs were visualized first, followed by a second application of FISH probes using the *B. rapa* centromere-specific DNA probes CentBr1 and CentBr2. To integrate the cytogenetic and genetic maps of *B. oleracea* and *B. napus*, we also hybridized the same set of *B. rapa* BAC clones to *B. oleracea* and *B. napus* to find corresponding homoeologous chromosomes between A and C genomes.

Selection of BAC-FISH probes containing repetitive elements: While screening *B. rapa* BAC clones during the selection of chromosome-specific BACs, we found some BAC clones containing repetitive sequences that detected more than one pair of chromosomes or had polymorphism for strength of signal. BACs of this type included KBrB072L17, which detected signals on eight pairs of chromosomes of *B. rapa*, and BAC KBrH092N2, which detected two pairs of chromosomes of *B. rapa* with differing signal strengths. In addition, BAC BNIH 123L05 from a *B. napus* library (Isobel Parkin, personal communication) was used to identify C-genome chromosomes because it gave a GISH-like pattern similar to that seen by Alix *et al.* (2008).

Selection of repetitive DNA sequence probes: In addition to the three BACs containing repetitive elements, four repetitive DNA sequences were used for karyotyping: 45S rDNA, 5S rDNA, CentBr1, and CentBr2. Inserts of plasmids containing 45S and 5S rDNA were PCR-amplified using M13 forward and reverse primers as described previously (Kato *et al.* 2004; Lamb and Birchler 2006). CentBr1 and CentBr2 have been previously characterized (Lim *et al.* 2005, 2007). The CentBr1 centromeric repeat was PCR-cloned using forward primer 5′-GAATAGCACAGCTTCATCGTCGTTCC-3′ and reverse primer 5′-CTGGGAAACTGTAATCACCTGATCTGAAA-3′. The CentBr2 centromeric repeat was PCR-cloned using forward primer 5′-GGGAATATGACACCTTCTTTGTCATTCT-3′ and reverse primer 5′-CAGGAAAACTGGGATCACCTGATTTAAAT-3′.

Slide preparation and fluorescence *in situ* hybridization: Immature flower buds (~2 mm long) were harvested from plants grown in the greenhouse for mitotic and meiotic chromosome spreads. Flower buds were treated with nitrous oxide gas for 1 h (Kato *et al.* 2004). Treated buds were fixed in ice-cold 90% acetic acid for 10 min and stored in 70% ethanol at −20° until used. Slides were prepared following the enzyme maceration method of Kato *et al.* (2004). DNA from BACs and repeated sequences were labeled with fluorescein-12-dUTP, Cy3-dCTP, and Cy5-dUTP or simultaneously with fluorescein-12-dUTP and Cy3-dCTP (Perkin Elmer Life Sciences, Boston, MA) using nick translation as previously described (Kato *et al.* 2004). FISH was performed following the method of Kato *et al.* (2004) with slight modifications (Lamb and Birchler 2006). The chromosome preparations were reused for the second time for FISH detection with CentBr1 and CentBr2 centromeric tandem repeat probes (Lim *et al.* 2005). The used slides were stripped by washing with 2× SSC containing 70% formamide at 70° for 2 min and dehydration by dipping the slides in 95% alcohol. Following hybridization and washes, a drop of Vectashield mounting medium containing DAPI (H-1200; Vector Laboratories, Burlingame, CA) was applied, and the cells were covered with a 24- × 50-mm cover glass. Visualization was performed using an Olympus BX61 fluorescent microscope with a 60× plan apo oil immersion lens, and digital images were captured using the Olympus Microsuite 5 software package (Olympus, Center Valley, PA). Images were cropped, size adjusted, and contrast optimized using only functions affecting the whole image with Adobe Photoshop 9.0.2 (Adobe Systems, San Jose, CA).

RESULTS

Development of chromosome-specific cytological markers for *B. rapa*: Using 20 *B. rapa* BAC clones that genetically mapped to opposite arms of the 10 *B. rapa* linkage groups, we verified that each pair of chromosome-specific BACs hybridized to the same pair of *B. rapa* chromosomes by initially using dual-color detection of FISH. The locations of the BAC clones on long or short arms were determined by a second application of FISH probes by using the *B. rapa* pericentromere-specific DNA probes CentBr1 and CentBr2 (Table 1, Figure 1). By performing 10 separate hybridizations, we identified which BACs were on the long or short arms of each chromosome (data not shown for these individual chromosome hybridizations). Relative to the karyotyping convention (shorter arms at top) and genetic map data, the orientations of linkage groups were concordant for chromosomes 3, 4, 5, 6, 7, and 9 but inverted for chromosomes 1, 2, 8, and 10 in *B. rapa* (Table 1). On the basis of the above results for 20 BACs, 16 BAC clones were chosen to be used in a pooled BAC-FISH karyotype probe mix to simultaneously identify all 10 chromosome pairs in three-color FISH on mitotic chromosomes (supporting information, Table S1). Using the distribution of pattern and the color of signals, all chromosome pairs of *B. rapa* ssp. *pekinensis* inbred line Chiifu were readily identified (Figure 1A).

Development of a standardized karyotype of *B. rapa*, *B. oleracea*, and allopolyploid *B. napus*: While screening *B. rapa* BAC clones during the selection of chromosome-specific BACs, two BAC clones, KBrB072L17 and KBrH092N2, were found to hybridize to more than two pairs of chromosomes, suggesting that these BACs contained repetitive sequences that had the potential to be good karyotype markers for Brassica species. BAC clone KBrB072L17 hybridized to eight pairs of chromosomes in *B. rapa*, with two pairs of strong signals, four pairs of medium signals, and two pairs of faint signals on mitotic chromosomes in both IMB218 (Figure 1B, green) and Chiifu (Figure 1E, green). KBrB072L17 also hybridized to several *B. oleracea* chromosomes (three pairs of strong signals, four pairs of medium signals, and three pairs of faint signals). FISH mapping on pachytene chromosomes revealed that these loci were located at the distal ends of several chromosomes on both diploid species (data not shown). The second BAC clone, KBrH092N2, yielded two pairs of strong signals on mitotic chromosomes in *B. rapa*, but only one major pair in *B. oleracea*. On pachytene chromosomes, this

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TABLE 1

Genetic markers and their anchored BACs used for integrating linkage and cytogenetic maps of *B. rapa*

Chromosome no.	BAC	BAC no.	Arm location of signal	Marker	Genetic position (cM)	Total map distance (cM)	Physical location in C genome
A1	KBrB042J11	1	Short	KS0540	83.3	99	C1
A1	KBrB066A08	2	Long	KS810	34		C1
A2	KBrB086G22	3	Short	KS50460	94.9	128	C2
A2	KBrH004D11	4	Long	KS50164	6.1		C2
A3	KBrB085J21	5	Long	KS40400	16.7	178	C7
A3	KBrH117M18	6	Long	KR50163	126.7		C3
A4	KBrB056C05	7	Short	KS31030	26	115	—
A4	KBrH009I04	8	Long	KS20840	78		C4
A5	KBrB001C24	9	Short	KS30340	47.1	124	C5
A5	KBrH033J07	10	Long				C4
A6	KBrB022P06	11	Short	KS10980	3.9	125	C6
A6	KBrH003P24	12	Long	KR30750-2a	117		C7
A7	KBrH049N07	13	Short			127	—
A7	KBrH052E24	14	Long				C6
A8	KBrB019A15	15	Long	KS210	102.6 (69.4)	115	C8
A8	KBrB048L11	16	Long	KR40760-3	67.9 (84.2)		C3
A9	KBrB043F18	17	Short	KS10050	6.8	193	C8
A9	KBrB022L12	18	Long	KS51160	134.3		C9
A10	KBrH053G06	19	Short	At5ILL1	53.2	83	—
A10	KBrH80A08	20	Long	KS50166	13.4		C9

signal was located on knob-like heterochromatin at several locations in *B. rapa* (data not shown).

The detailed chromosomal locations of the multiple hybridization signals from the BAC clones KBrB072L17 and KBrH092N24 were determined for *B. rapa*, *B. oleracea*, and *B. napus* by using dual-color FISH and different chromosome-specific BACs. For example, metaphase chromosomes of *B. rapa* Chiifu probed with KBrB072L17 (green) and chromosome 8 specific BAC clone KBrB019A15 (red) demonstrated that one faint KBrB072L17 locus was located on the short arm of chromosome 8 (Figure 1E). Using this dual-color FISH approach, we confirmed the location of each of the other KBrB072L17 loci on chromosomes 1S, 2S, 4L, 5S, 5L, 6S, 6L, 8S, and 9L. The same chromosomal location pattern was observed in both *B. rapa* lines (Chiifu and IMB218), as well as in the A genome of *B. napus* (Stellar). A similar approach was used to determine the locations of the multiple signals of KBrB072L17 in *B. oleracea* and the C genome of *B. napus,* since *B. rapa* chromosome-specific BACs also hybridize to homoeologous regions of *B. oleracea* and the C genome of *B. napus* (Bohuon *et al.* 1996; Lysak *et al.* 2005; Parkin *et al.* 2005). KBrB072L17 signals were located on chromosome 1S, 1L, 2S, 2L, 4S, 4L, 5S, 5L, 9S, and 9L of *B. oleracea*. KBrH092N24 loci are located on chromosomal arms 2L and 7L of *B. rapa* (Figure 2A, red) and 6L of *B. oleracea* (Figure 2C, red). The different signal strengths and chromosomal locations for the various hybridization sites of KBrB072L17 and KBrH092N24 provided robust and consistent cytological markers for *B. rapa*, *B. oleracea*, and *B. napus*.

A standardized karyotype of *B. rapa*, *B. oleracea*, and allopolyploid *B. napus* was created by multi-color FISH using the KBrB072L17 and KBrH092N24 repetitive element-containing BAC clones described above in concert with previously known repetitive DNA sequences: 45S rDNA and 5S rDNA and CentBr1 and CentBr2 (two pericentromeric 176-bp satellite repeats; Lim *et al.* 2005, 2007). All somatic chromosomes showed distinctive karyotype patterns among the three Brassica species (Figures 2, A–F). To reconfirm the identity of the A and C genomes in *B. napus*, a second hybridization step was used to identify C-genome chromosomes by using *B. napus* BAC BNIH 123L05 as a probe. By verifying the location of each of these repetitive elements in *B. rapa*, *B. oleracea*, and allopolyploid *B. napus* by using the 16 chromosome-specific *B. rapa* BACs, a diagnostic repeat-element karyotype probe mix that was easier to use than the chromosome-specific karyotype probe mix was constructed.

Integration of the cytogenetic and genetic linkage maps and identification of homoeologous chromosomes between A and C genomes in *B. rapa*, *B. oleracea*, and allopolyploid *B. napus*: Using 20 chromosome-specific BAC markers and repeat sequences, the distinctive staining patterns of repeated sequences corresponding to their genetic linkage groups were integrated in *B. rapa.* To integrate the cytogenetic and genetic maps of *B. oleracea* and *B. napus*, we also used the same set of *B. rapa* BAC clones to hybridize to the *B. oleracea* and *B. napus* chromosomes. From the information of the genetic maps of *B. oleracea* and *B. napus*, these BAC-FISH cytogenetic markers not only integrated the two maps,

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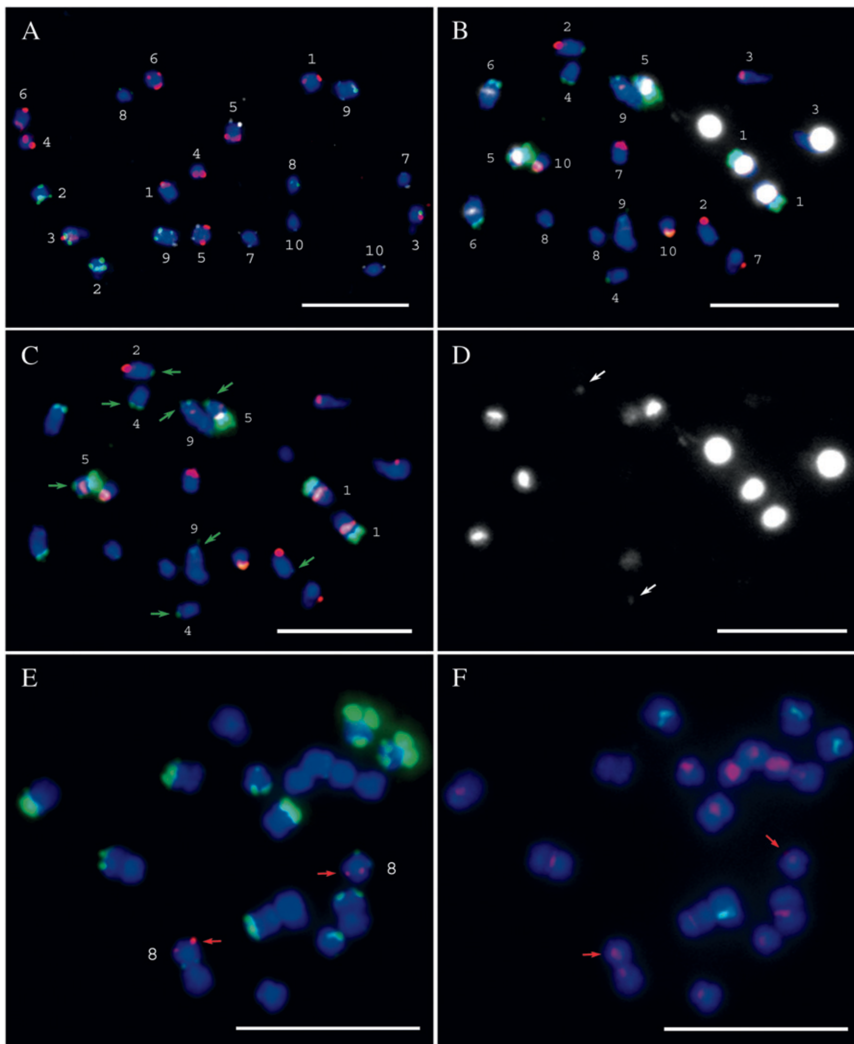

FIGURE 1.—Somatic karyotype of *B. rapa*. (A) Simultaneous FISH of a 16-BAC probe to the *B. rapa* Chiifu mitotic metaphase chromosome spread. The patterns of signals enable FISH-based recognition of each chromosome pair and associated specific linkage groups. Each chromosome number corresponds to a linkage group. (B–D) Somatic chromosome karyotyping of *B. rapa* IMB218 probed with the FISH mixture: 45S (white), 5S (light green), BAC clone KBrB072L17 (green), and KBrH092N24 (red). (C) Somatic chromosome karyotyping of *B. rapa* IMB218 shows the signals of 5S (light green), BAC clones KBrB072L17 (green), and KBrH092N24 (red). Arrows show the small loci of KBrB072L17. (D) Somatic chromosome karyotyping of *B. rapa* IMB218 shows 45S (white). Arrows show the smallest 45S locus on A4. (E) FISH with KBrB072L17 (green) and chromosome 8-specific BAC clone KBrB019A15 (red) on the metaphase chromosomes of *B. rapa* Chiifu. (F) The same cell as shown in E reprobed with CentBr1 (red) and CentBr2 (green) showed the position and organization of the centromere. All scale bars, 10 μm.

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but also showed corresponding homoeologous chromosomes between A and C genomes. For example, the BAC clone KBrH038M21 from the long arm of chromosome A3 hybridized to *B. rapa*, *B. oleracea*, and *B. napus* chromosomes (Figure 3). One pair of very strong BAC signals was detected on *B. oleracea* (Figure 3C) and on the C genome of *B. napus* (Figure 3F). This chromosome pair was named C3 on the basis of the signals and genetic information of *B. napus*. It is interesting that we did not detect any strong signals on *B. oleracea* using BACs from the short arms of 4S, 7S, and 10S of *B. rapa*. Those results were confirmed on resynthesized *B. napus* and natural *B. napus* (Stellar), and strong signals were detected on the A genome (data not shown). In *B. napus*, no significant homoeologous chromosome rearrangements were detected according to repeated sequence signals located on end of chromosomes. However, we did detect one large chromosomal rearrangement on the long arm of A7, which contained red signals specific to the C genome. We integrated chromosomal painting of sequence repeats, BAC-FISH, and analysis of centromere organizations in *B. rapa* (Figure 4), *B. oleracea* (Figure 5), and *B. napus* (Figure 6).

Idiograms summarize the karyotypes of *B. rapa* Chiifu (Figure 7A), *B. rapa* IMB218 (Figure 7B), *B. oleracea* TO1000 (Figure 7C), and *B. napus* Stellar (Figure 7D). The features of chromosomes A1–A10 correspond to linkage groups A1–A10 of *B. rapa* or the A-genome linkage groups of *B. napus*. Similarly, chromosomes C1–C9 correspond to linkage groups 1–9 in *B. oleracea* or to C-genome linkage groups in *B. napus*. Descriptions of the probes that hybridize to each chromosome, comparisons between homoeologous A and C chromosomes of diploid *B. rapa* and *B. oleracea*, and comparisons between subgenomes A and C across *B. napus* chromosomes are summarized below.

Chromosome A1: The largest 5S and the second largest 45S signals are on the pericentromeric region of its long arm. Strong signals from KBrB072L17 are at the tip of the short arm. A1 appears to be homoeologous to C1.

Chromosome A2: The strongest signals of KBrB092N24 are localized near the end on the long arm of A2. Very small KBrB072L17 signals are found at the tip of the short arm. A2 appears to be homoeologous to C2.

Chromosome A3: The largest nucleolar organizing region (NOR) signals and 5S signals are present in this

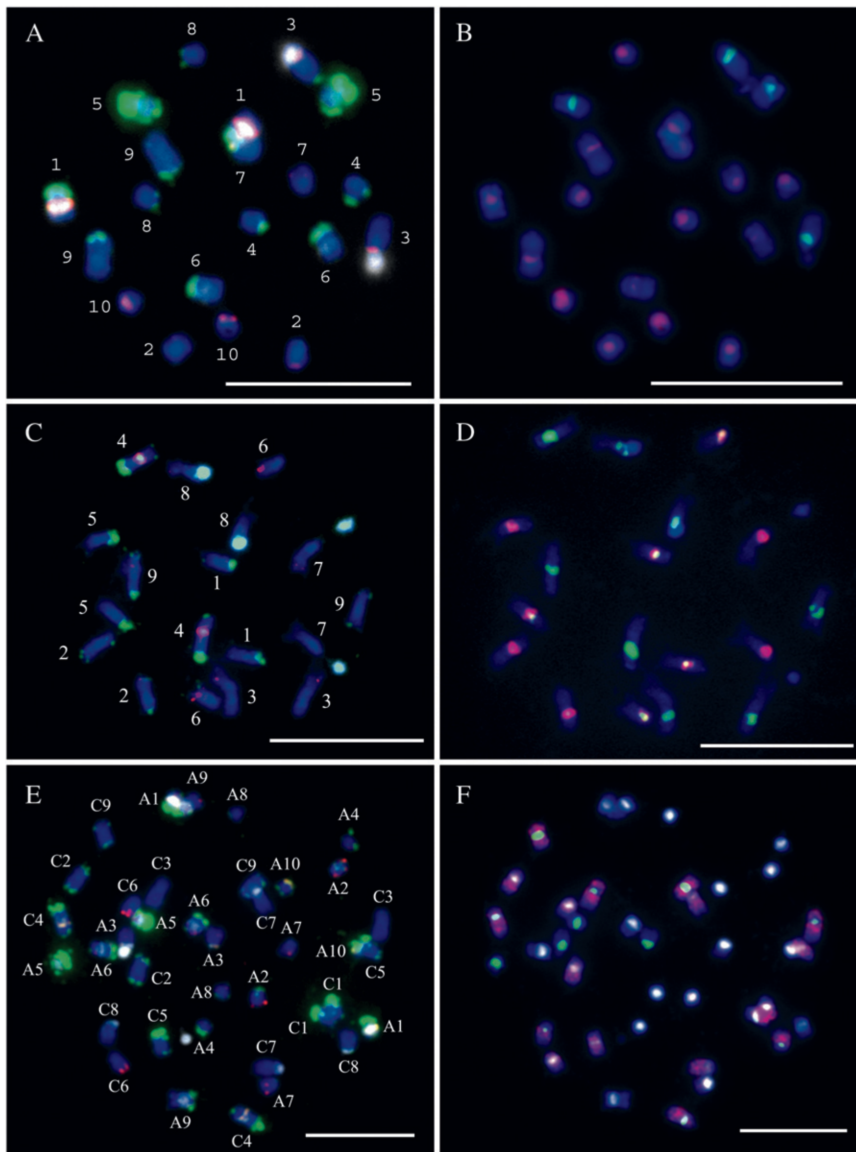

Figure 2.—Karyotyping of *B. rapa*, *B. oleracea*, and *B. napus*. (A, C, and E) Repeated sequences 45S (white), 5S (light green), and BAC KBrH092N24 (red) and KBrB072L17 (green) containing repeated sequences to make karyotypes of *B. rapa*, *B. oleracea*, and *B. napus*. (B, D, and F) The same cell of A, C, and E, respectively, reprobed with CentBr1 (red) and CentBr2 (green) shows the position and organization of the centromere. (A and B) *B. rapa* Chiifu. (C and D) *B. oleracea*. (E and F) *B. napus* (Stellar). All scale bars, 10 μm.

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chromosome. A3 displays CentBr2 pericentromere repeats. The long arm of A3 is homoeologous to the long arm of C3. The middle of A3 is homoeologous to the region on the very end of C7.

Chromosome A4: A medium strength KBrB072L17 signal is present at the end on the long arm of A4. The smallest 45S signals are detected in the pericentromeric region on the short arm of A4 in the subspecies IMB218 (Figure 1D), but absent on A4 of *B. rapa* Chiifu and *B. napus*. The long arm of A4 is homoeologous to the long arm of C4. We did not find the corresponding homoeologous region of its short arm in the C genome.

Chromosome A5: The strongest KBrB072L17 signals are present at the end of the short arm on A5, and medium strength KBrB072L17 signals are localized at the end on its long arm. The smallest 45S signals are located within the pericentromeric region of the long arm on A5 in *B. rapa* Chiifu. The short and long arms of A5 are homoeologous to the short arm of C5 and C4, respectively.

Chromosome A6: Relatively strong KBrB072L17 signals are present at the end on the short arm of A6. Medium strength 45S signals are present within the pericentromeric region on the long arm of A6. The short and long arms of A6 are homoeologous to the short arm of C6 and the long arm of C7, respectively.

Chromosome A7: There are signals of KBrB092N24 near the middle on the long arm of A7. A7 displays CentBr1 repeats. Although the short arm of A7 is syntenic with C7 according to genetic map data (Parkin *et al.* 2005), strong signals were not detected in the C genome using several BACs from this region of A7. The long arm of A7 is homoeologous to C6.

Chromosome A8: A faint KBrB072L17 signal is present at the end on the short arm of A8. The end and the middle of the long arm of A8 appear to be homoeolo-

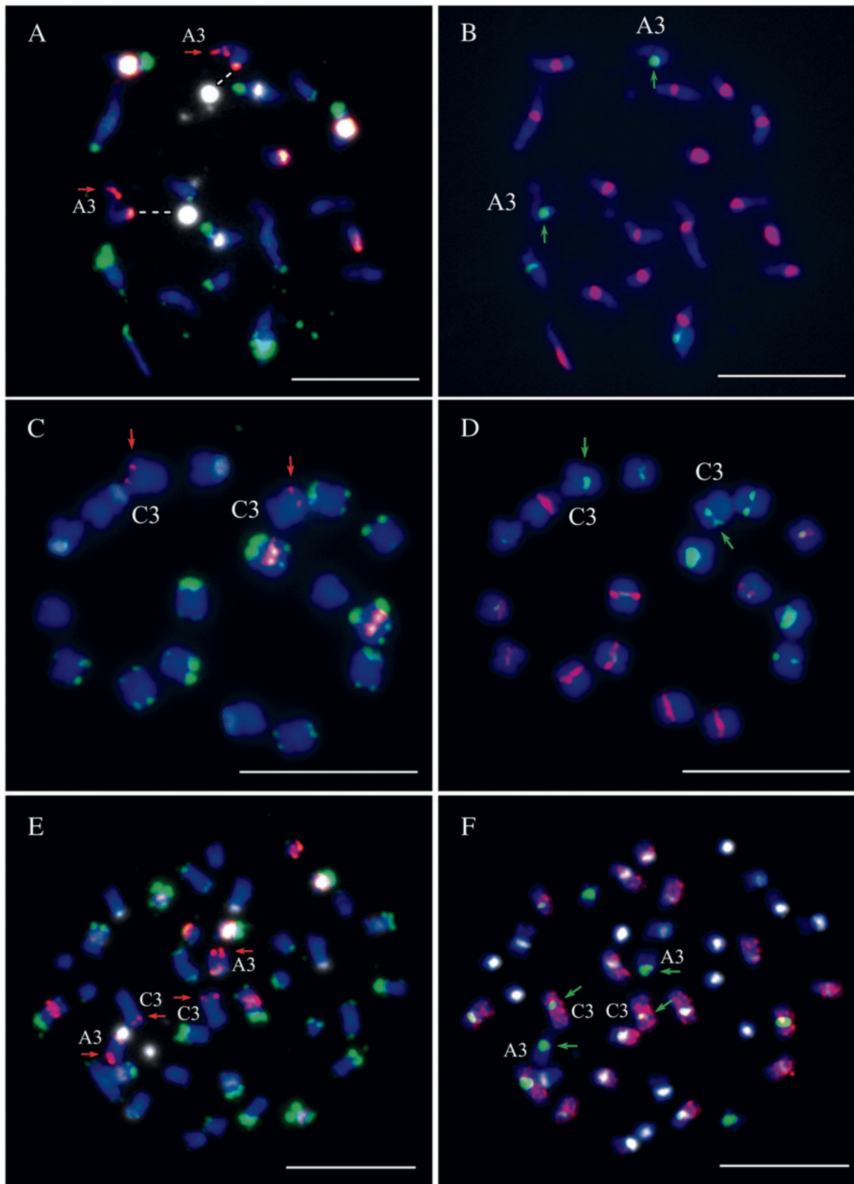

FIGURE 3.—Chromosome and arm-specific *B. rapa* BACs and repeated sequence integrate the cytogenetic and genetic maps of *B. rapa*, *B oleacea*, and *B. napus*. (A) BAC KBrH038M21 (red) located on long arm of A3 of *B. rapa*. This chromosome has large 45S signals (white signal, connected by white dashes) and 5S signals on the end of the short arm but no KBrB072L17 (green). (B) The same cell as shown in A reprobed with CentBr1 (white), CentBr2 (green), and the centromere of A3 is present mainly in the CentBr2 repeated sequence. (C). *B. rapa* BAC KBrH038M21 shows strong signals on the long arm of one pair of *B. oleracea* chromosomes. This chromosome is named C3 according to genetic mapping data, and it lacks KBrB072L17 and NOR signals. (D) The same cell as shown in C reprobed with CentBr1 (white) and CentBr2 (green). The centromere of C3 is present mainly in the CentBr2 repeated sequence. (E) *B. rapa* BAC KBrH038M21 was located on natural *B. napus* (Stellar). Arrows show the homeologues A3 and C3. (F) The same cell shown in E reprobed with CentBr1 (white), CentBr2 (green), and BAC BNIH 123L05 containing C-genome-specific repeated sequences (red). Both centromeres of A3 and C3 contain a CentBr2 repeated sequence. All scale bars, 10 μm.

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gous to the short arm of C3 and the middle of the long arm of C8, respectively.

Chromosome A9: A medium-strength KBrB072L17 signal is present at the end on the long arm of A9. A small 45S locus is present in the pericentromeric region on the long arm of A9 in both *B. rapa* Chiifu and *B. rapa* IMB218. A small 5S locus is present in the pericentromeric region on the long arm of A9 in *B. rapa* IMB218, but is absent in *B. rapa* Chiifu. *B. napus* has a medium-size 45S locus and a small 5S locus on this chromosome. A9 is the largest chromosome in the A genome. The short and the long arms of A9 are homoeologous to the long arm of C8 and the long arm of C9, respectively.

Chromosome A10: A 5S locus is located on the short arm of A10. A10 is the smallest chromosome. A very faint KBrB072L17 signal is present at the end of its long arm in *B. napus*. The long arm of A10 is homoeologous to the short arm of C9.

Chromosome C1: This chromosome contains a strong and a faint KBrB092N24 signal on short and long arms, respectively. C1 displays both CentBr1 and CentBr2 repeats.

Chromosome C2: Both short and long arms of C2 have medium-strength KBrB072L17 signals. C2 displays CentBr1 repeats.

Chromosome C3: No repeat sequences signals were detected on either arms of this chromosome. C3 is the largest chromosome in the C genome and its centromere contains CentBr2 repeats.

Chromosome C4: A strong and a medium-strength KBrB072L17 signal are present in C4 at the end of its short and long arm, respectively. A 5S locus is located on its long arm near the centromere. The centromere of C4 contains CentBr2 repeats in *B. oleracea*, but both CentBr1 and CentBr2 repeats are visualized in *B. napus* C4.

Chromosome C5: This chromosome contains a strong and a faint KBrB072L17 signal on the short and long

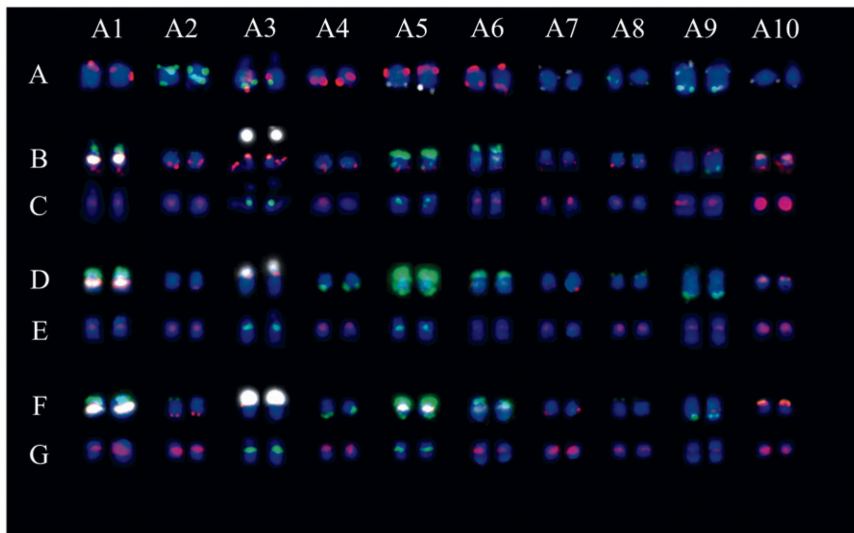

Figure 4.—Integrated somatic chromosome karyotype of *B. rapa*. (A) Simultaneous FISH of a 16-BAC probe to *B. rapa* Chiifu. Chromosomes cut out from the same cell as shown in Figure 1A. (B) Chromosome-specific BAC markers (red) from 10 linkage group to identify the distinctive FISH staining patterns on different chromosomes of *B. rapa* (Chiifu) [45S (white), 5S (light green), and BAC clone KBrB072L17 (green)]. BACs used for identifying chromosomes 1–10 are KBrB066A08, KBrH004D11, KBrH038M21, KBrH009I04, KBrH033J07, KBrH003P24, KBrH052E24, KBrB048L11, KBrB043F18, and KBrH80A08. Chromosomes were cut out from different FISH hybridizations. (C) The same chromosome as shown in B reprobed with CentBr1 (red) and CentBr2 (green), which shows the position and organization of the centromere on each chromosome. (D) *B. rapa* Chiifu probed with the FISH mixture: 45S (white), 5S (light green), BAC clone KBrB072L17 (green), and KBrH092N24 (red). (E) The same chromosome as shown in D reprobed with CentBr1 (red) and CentBr2 (green). (F) *B. rapa* IMB218 probed with the FISH mixture: 45S (white), 5S (light green), BAC clone KBrB072L17 (green), and KBrH092N24 (red). (G) The same chromosome as shown in F reprobed with CentBr1 (red) and CentBr2 (green).

arms, respectively. The only observed difference in C5 between *B. oleracea* and *B. napus* is the organization of the centromere, in which *B. oleracea* contains both CentBr1 and CentBr2 repeats and *B. napus* contains only faint CentBr1 signals. In *B. oleracea*, the signal pattern of C5 is similar to C1, but some differences do exist between them. C5 has stronger KBrB072L17 and CentBr1 signals and smaller CentBr2 signals compared to C1.

Chromosome C6: KBrB092N24 signals are present on the long arm of C6. The C6 centromere contains both CentBr1 and CentBr2 signals in *B. oleracea*. In *B. napus*, mainly CentBr1 signals were detected in the centromere of C6.

Chromosome C7: This chromosome has a 45S locus on the end of its short arm and CentBr1 repeats. *B. oleracea* has stronger 45S signals on C7 compared to *B. napus*.

Chromosome C8: This chromosome has a 45S locus on the end of its short arm and CentBr2 repeats. *B. oleracea* has stronger 45S signals on C8 compared to *B. napus*.

Chromosome C9: C9 has medium-strength KBrB092N24 signals on the ends of both arms and CentBr2 repeats.

In sum, we were able to find homoeologous regions from all chromosome arms of *B. rapa* to *B. oleracea* except for the short arms of A4, A7, and A10.

DISCUSSION

Robust molecular cytogenetic karyotypes established for *B. rapa*, *B. oleracea*, and *B. napus*: Numerous molecular cytogenetic studies have reported karyotypes in diploid and amphidiploid Brassica species on mitotic metaphase complements and meiotic prophase (pachytene) chromosomes (*e.g.*, Armstrong *et al.* 1998, Fukui *et al.* 1998; Snowdon *et al.* 2002; Koo *et al.* 2004; Lim *et al.* 2005, 2007; Maluszynska and Hasterok 2005; Howell *et al.* 2008; Kim *et al.* 2009; Mun *et al.* 2009). Among the crop species in the Brassicaceae, the *B. rapa* genetic, physical, and cytogenetic maps have begun to be integrated by BAC-FISH (Koo *et al.* 2004; Lim *et al.* 2005, 2007; Yang *et al.* 2007; Kim *et al.* 2009; Mun *et al.* 2009; Xiong *et al.* 2010). However, to our knowledge, a complete karyotype analysis that reliably distinguishes each chromosome in *B. oleracea* and *B. napus* has not been reported. The primary obstacle has been that hybridization of *B. oleracea* BACs to *B. oleracea* chromosomes often gives little information because the probes hybridize to multiple locations of the genome due to the presence of repetitive elements (Howell *et al.* 2002, 2005, 2008; Kwon *et al.* 2007; Alix *et al.* 2008), similar to what was reported in the BAC-FISH studies of Allium (Suzuki *et al.* 2001) and maize (Koumbaris and Bass 2003; reviewed in Danilova and Birchler 2008). In this study, robust karyotypes of *B. rapa*, *B. oleracea*, and *B. napus* were established. Several distinct advantages exist in our molecular cytogenetic karyotypes compared with previously published karyotypes. First, every mitotic chromosome of both A and C genomes can be readily and unambiguously identified by distinct signal features. We also integrated previously available genetic maps with our cytogenetic maps of *B. rapa*, *B. oleracea*, and *B. napus*. Second, we used BAC clones from *B. rapa* to identify the homoeologous regions in the related species *B. oleracea*. Using this method, we were able to identify homoeologous regions between *B. rapa* and *B. oleracea* and between the A and C genomes in the allopolyploid *B. napus*. Third, we introduce a new chromosome nomenclature system that follows the international linkage group system for Brassica (Parkin *et al.* 2005; Kim *et al.*

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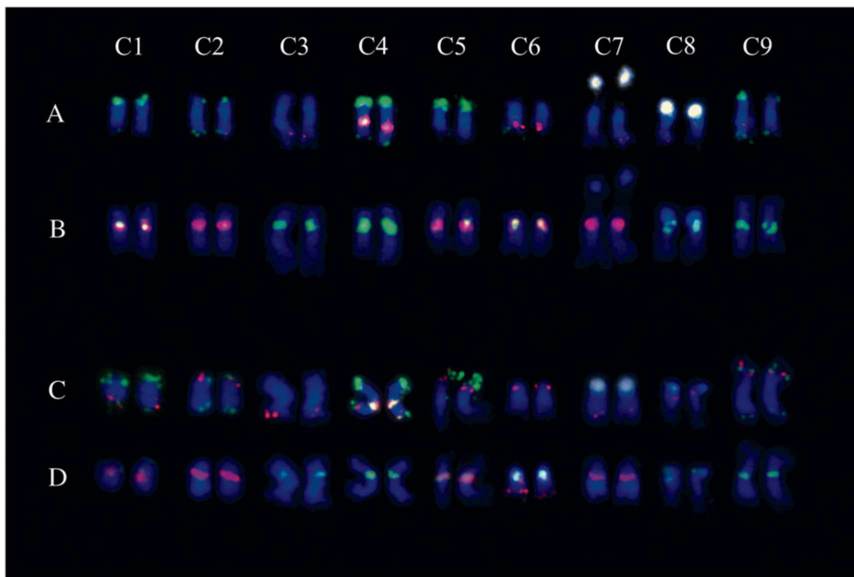

FIGURE 5.—Integrated somatic chromosome karyotype of *B. oleracea*. (A) *B. oleracea* TO1000 probed with the FISH mixture: 45S (white), 5S (light green), BAC clone KBrB072L17 (green), and KBrH092N24 (red). (B) The same chromosome as shown in A reprobed with CentBr1 (red) and CentBr2 (green). (C) Chromosome-specific BACs from *B. rapa* (red) used to identify the distinctive FISH staining patterns of homoeologous chromosome of *B. oleracea*. BACs used from chromosome C1 to C9 are KBrB066A08, KBrB086G22, KBrH117M18, KBrH009I04, KBrB001C24, KBrB022P06, KBrH003P24, KBrB019A15, and KBrH80A08, and chromosomes are cut out from different cells. (D) The same chromosome as shown in C reprobed with CentBr1 (red) and CentBr2 (green).

2006; OSTERGAARD and KING 2008; see also http://www.brassica.info/resource/maps/lg-assignments.php) instead of previous traditional systems that use only chromosomal size parameters to assign a chromosome number (*e.g.*, LIM *et al.* 2005, 2007).

We found two unique BACs, KBrB072L17 and KBrH092N24, which contain repeated sequences and serve as excellent chromosome markers for two reasons. First, unlike the typical markers (*e.g.*, 45S and 5S rDNA) that may exhibit polymorphisms in number, signal strength, and chromosomal distribution in the same species (KOO *et al.* 2004), the distribution features of the repeated sequences from KBrB072L17 and KBrH092N24 are very stable in both A or C genomes among different Brassica species. We also observed polymorphisms for NOR loci within the A genome among Chiifu, IMB218, and *B. napus*, as well as within the C genome between *B. oleracea* and *B. napus*. In addition, the composition of centromeric repeats was polymorphic within the C genome between *B. oleracea* and *B. napus*. These results suggested that methods of karyotype analysis using these two unique BACs might be also suitable for different subspecies with A or C genomes. Future work will involve cloning these repeats and localizing them in other species of the genus Brassica. Second, these markers serve as excellent cytological tools for detecting chromosomal rearrangements because they give distinct signal patterns observable at chromosomal end locations on various chromosomes.

Brassica karyotype analysis as a tool to improve our understanding of A- and C-genome evolution: The exact cytological characterization of Brassica addition, substitution, and particularly introgression lines has been restricted by the lack of distinct karyological features that can be readily identified in metaphase preparations (SNOWDON 2007). Our molecular cytogenetic tool kit will facilitate the development and characterization of new cytological stocks in Brassica. The described karyotype analysis will allow researchers to correctly identify chromosomes carrying agriculturally significant genes introduced into Brassica cultivars (NAVABI *et al.* 2010). In addition, these karyotypes will aid the ongoing sequencing of Brassica genomes by integrating the genetic, physical, and cytogenetic maps, as we have demonstrated for chromosome A7 (XIONG *et al.* 2010). Because the genus Brassica shares a whole-genome triplication event (LUKENS 2004; LYSAK *et al.* 2005; PARKIN *et al.* 2005; YANG *et al.* 2006; MANDAKOVA and LYSAK 2008; CHEUNG *et al.* 2009; TRICK *et al.* 2009), repetitive sequence blocks and molecular fingerprinting errors have made it difficult to assemble the physical map and BAC contigs in Brassica (GREGORY *et al.* 1997; MUN *et al.* 2008). Using the karyotype approach presented here, individual BAC clones can be accurately localized in detail to chromosomes and linkage groups using FISH. Because the Brassica genomes have undergone ancient whole-genome duplications that are detectable using Arabidopsis BAC clones in a comparative chromosome painting approach (LYSAK *et al.* 2005; LYSAK 2009), hybridization conditions were optimized to select against visualization of those paralogs.

Comparative genetic mapping studies have demonstrated that minor recombination has occurred between the A and C genomes since the spontaneous origin of *B. napus* (PARKIN *et al.* 1995; SHARPE *et al.* 1995; OSBORN *et al.* 2003a; PIQUEMAL *et al.* 2005; UDALL *et al.* 2005). Furthermore, cytogenetic studies using GISH have confirmed that the A and C genomes have largely remained distinct in *B. napus* (HOWELL *et al.* 2008). These studies imply that the A and C genomes should be largely intact in the amphidiploid *B. napus*. Using BAC-containing C-genome-specific repeated sequences, homoeologous recombination between the A and C

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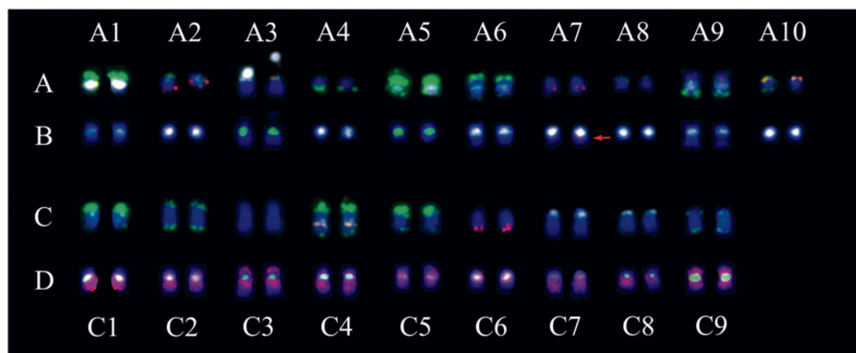

Figure 6.—Somatic chromosome karyotype of *B. napus*. (A and C) *B. napus* probed with the FISH mixture: 45S (white), 5S (light green), BAC clone KBrB072L17 (green), and KBrH092N24 (red). (A) The A-genome chromosomes numbered following genetic linage groups 1–10. (C) The C-genome chromosomes numbered following genetic linage groups 11–19. (B and D) The same cell in A and C reprobed with CentBr1 (white), CentBr2 (green), and BAC BNIH 123L05 containing C-genome-specific repeated sequences (red). Arrow shows the red signals on the long arm of A7.

genomes was detected only on chromosome A7 in *B. napus* Stellar, consistent with the previously published data (Osborn *et al.* 2003a; Howell *et al.* 2008).

The *Arabidopsis thaliana* genome has been subdivided into 21 conserved segments (*i.e.*, genomic blocks) (Parkin *et al.* 2005), which have been duplicated and rearranged to form the entire *B. napus* genome (Schranz *et al.* 2006). Our molecular cytogenetic map was able to identify the corresponding homoeologous chromosomes or regions between the A and C genomes, and we optimized hybridization conditions to avoid detecting ancient paralogous segments indicative of ancient polyploidy events (Lysak *et al.* 2005; Lysak 2009). Most of our physical mapping results were consistent with the published genetic map of *B. napus* (Parkin *et al.* 2005). However, small differences between the genetic map and our FISH results were observed. In addition, we were unable to identify three homoeologous regions on the C genome using BAC clones from the short arms of A4, A7, and A10. These inconsistencies may be due to the deletions of homoeologous regions on the C genome after the A and C genomes diverged. Both chromosome A8 and its homoeologous chromosome C8 are syntenous with Arabidopsis C1C, C4B, and C1AB in *B. napus* (Parkin *et al.* 2005). However, we detected strong signals on the short arm of C3 instead of C8 in *B. oleracea* and *B. napus* (Figure 7D) by using several BACs (data not shown), including KBrB048L11, which came from the C4B block of A8 in *B. rapa* to find the homoeologous region on C genome. According to the genetic map of Parkin *et al.* (2005), both A3 (N3) and its homoeologous chromosome C3 (N13) contained another duplicated C4B block in *B. napus*. These results suggested that an ancient chromosome rearrangement occurred between the two duplicated regions of C4B in either the A or C genomes after the diversification from a common ancestor.

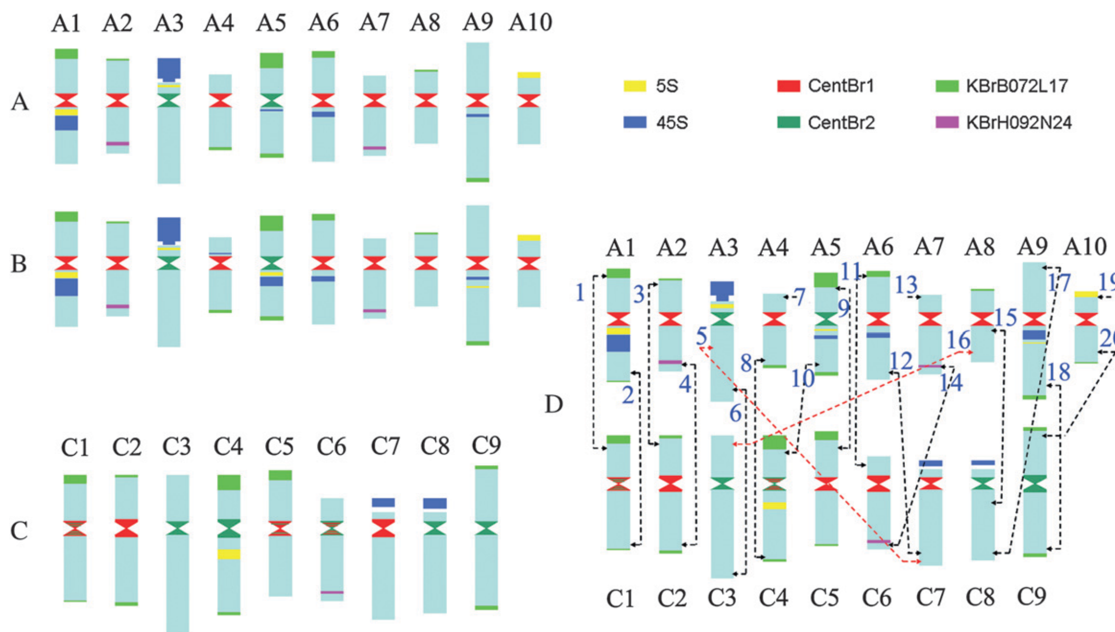

Figure 7.—Idiograms of *B. rapa*, *B. oleracea*, and *B. napus*. (A) *B. rapa* subspecies Chiifu. (B) *B. rapa* subspecies IMB218. (C) *B. oleracea* TO1000. (D) *B. napus* Stellar. Chromosomal arm-specific BACs used to identify the corresponding homoeologous chromosomes between the A and C genomes of *B. napus* are numbered following the scheme in Table 1.

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The future of plant molecular cytogenetics is promising in terms of both methods and applications (Kato *et al.* 2005; Walling *et al.* 2005; Lamb and Birchler 2006; Danilova and Birchler 2008; Guerra 2008; Pires and Hertweck 2008; Lysak *et al.* 2010; Xiong *et al.* 2010). The robust novel chromosomal painting technique developed here will assist the understanding of chromosome pairing, homoeologous recombination, and genome evolution in the genus Brassica and will facilitate new applied breeding technologies in this genus that rely upon identification of individual chromosomes. In particular, there are few allopolyploids where the homoeologous chromosomes can be tracked cytogenetically. Genetic mapping studies of domesticated *B. napus* cultivars find only a few chromosomal rearrangements among the homoeologous regions of the A and C genomes (Parkin *et al.* 1995; Sharpe *et al.* 1995; Osborn *et al.* 2003a; Udall *et al.* 2005); however, extensive chromosomal rearrangements are found in resynthesized *B. napus* (Song *et al.* 1995; Pires *et al.* 2004; Lukens *et al.* 2006; Gaeta *et al.* 2007; Cifuentes *et al.* 2010; Gaeta and Pires 2010; Pires and Gaeta 2010; Szadkowski *et al.* 2010). The FISH-based karyotyping system developed here is a powerful approach for probing chromosome structure, and our validated Brassica karyotyping tool kit may lead to other important applications such as the characterization of trisomics, translocation and inversion lines, and tracking chromosomes from interspecies crosses (Findley *et al.* 2010; Navabi *et al.* 2010).

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Karyotype and Identification of All Homoeologous Chromosomes of Allopolyploid *Brassica napus* and Its Diploid Progenitors

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TABLE S1

List of 16 BACs used for three-color FISH on mitotic chromosomes and Probe-Mixture Constitution

Chromosome no.	BAC	Arm location of signal	Fluorescence used	Signal Colors
A1	KBrB042J11	Short	Cy3 -dUTP and Cy5-dUTP	Red and white
A2	KBrB086G22	Short	fluorescein-12-dUTP	Green
	KBrH004D11	Long	fluorescein-12-dUTP	Green
A3	KBrB085J21	Long	Cy3 -dUTP	Red
	KBrH117M18	Long	fluorescein-12-dUTP	Green
A4	KBrH009I04	Long	Cy3 -dUTP	Red
A5	KBrB001C24	Short	Cy3 -dUTP	Red
	KBrH033J07	Long	Cy5 -dUTP	White
A6	KBrB022P06	Short	Cy3 -dUTP	Red
	KBrH003P24	Long	Cy3 -dUTP	Red
A7	KBrH049N07	Short	Cy5 -dUTP	White
	KBrH052E24	Long	Cy5 -dUTP	White
A8	KBrB019A15	Long	fluorescein-12-dUTP	Green
A9	KBrB043F18	Short	Cy5 -dUTP	White
	KBrB022L12	Long	fluorescein-12-dUTP	green
A10	KBrH80A08	Long	Cy5 -dUTP	White

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