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Repeated Polyploidy Drove Different Levels of Crossover Suppression between Homoeologous Chromosomes in *Brassica napus* Allohaploids [C] [W]

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Allopolyploid species contain more than two sets of related chromosomes (homoeologs) that must be sorted during meiosis to ensure fertility. As polyploid species usually have multiple origins, one intriguing, yet largely underexplored, question is whether different mechanisms suppressing crossovers between homoeologs may coexist within the same polyphyletic species. We addressed this question using *Brassica napus*, a young polyphyletic allopolyploid species. We first analyzed the meiotic behavior of 363 allohaploids produced from 29 accessions, which represent a large part of *B. napus* genetic diversity. Two main clear-cut meiotic phenotypes were observed, encompassing a twofold difference in the number of univalents at metaphase I. We then sequenced two chloroplast intergenic regions to gain insight into the maternal origins of the same 29 accessions; only two plastid haplotypes were found, and these correlated with the dichotomy of meiotic phenotypes. Finally, we analyzed genetic diversity at the *PrBn* locus, which was shown to determine meiotic behavior in a segregating population of *B. napus* allohaploids. We observed that segregation of two alleles at *PrBn* could adequately explain a large part of the variation in meiotic behavior found among *B. napus* allohaploids. Overall, our results suggest that repeated polyploidy resulted in different levels of crossover suppression between homoeologs in *B. napus* allohaploids.

INTRODUCTION

Meiosis is an obligatory process for all sexually reproducing organisms. This specialized type of cell division is essential to produce gametes, ensure genome stability throughout sexual life cycles, and generate diversity within species by creating new chromosome/allele combinations. For all these outcomes, the exclusive formation of crossovers (COs) between homologous chromosomes is required (Hamant et al., 2006). Our knowledge of the genes and mechanisms involved in meiotic recombination and CO formation in plants has received a boost in the last 6 years with the use of *Arabidopsis thaliana*, rice (*Oryza sativa*), and maize (*Zea mays*) as model systems (Mezard et al., 2007; Mercier and Grelon, 2008). However, little is known about natural variation in recombination rates within species (Säll, 1990; Sanchez-Moran et al., 2002; Anderson et al., 2003; Esch et al., 2007; Bovill et al., 2008), which is an important issue for understanding how recombination is regulated in the wild.

The existence of natural variability for CO regulation is particularly relevant in polyploid species, considering that most have multiple origins and that genetic systems restricting CO to homologs are required. Recurrent polyploidy is known to be the rule rather than an exception. Most nascent polyploid species should be considered as a set of genetically variable lineages produced from distinct diploid progenitors that can subsequently produce novel genotypes through hybridization and recombination (Soltis and Soltis, 1999). As these new polyploid species are not completely isolated from their diploid progenitors, the variability present in the diploids can continue to be incorporated into the polyploids. This may subsequently lead to different genetic/epigenetic changes, which can further expand the range of phenotypes. These eventualities are yet to be addressed experimentally (e.g., Dubcovsky and Dvorak, 2007; Koh et al., 2010), and little is known about the consequences of recurrent polyploidy on the establishment of polyploid species, in particular for processes that are directly relevant to natural selection, such as meiosis.

Proper chromosome segregation is a demanding process in polyploid species, including those of hybrid origin (i.e., allopolyploids). These species have more than two complete sets of chromosomes related by ancestral homology (so-called homoeologs). Homoeologs are usually still able to form COs during meiosis but must be sorted to produce viable and balanced

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gametes. Suppression of COs between homoeologous chromosomes is thus required to ensure fertility. Current understanding is that this process is genetically determined in many allopolyploids and usually subject to polygenic regulation (Jenczewski et al., 2003; Cifuentes et al., 2010). However, it is not known whether recurrent polyploidy drives variation in the determinants of CO between homoeologous chromosomes in allopolyploid species.

Wheat is the main model for which some data are available. Common bread wheat (*Triticum aestivum*; 2n = 6x = 42; genome formula AABBDD) is the product of two successive polyploidization events (2x→4x then 4x→6x); it arose from at least two maternal lineages (Hirosawa et al., 2004), had two genetically distinct D-genome progenitors (Caldwell et al., 2004), and captured a large portion of the natural genetic diversity present in its tetraploid ancestor (Dubcovsky and Dvorak, 2007). In both tetraploid and hexaploid wheat, the main locus responsible for exclusive homologous CO formation is *Ph1* (Riley and Chapman, 1958; Feldman, 1966; Sears, 1976; Giorgi, 1978), which was recently defined to a region containing a cluster of cyclin-dependent kinase-related genes interrupted by a heterochromatin segment (Griffiths et al., 2006; Al-Kaff et al., 2008). This idiosyncratic structure is apparently conserved among polyploid wheat species (Griffiths et al., 2006), which is in contrast with the slight variability observed for CO suppression between homoeologous chromosomes in both tetraploid (Ozkan and Feldman, 2001) and hexaploid (Martinez et al., 2005) wheat species.

The limited data available on *Brassica napus* suggest that the situation could be different in this species, although it evolved roughly in the same way as wheat. *B. napus* (AACC; 2n = 38) is a young allopolyploid species with multiple origins (i.e., a polyphyletic species) that formed by repeated interspecific hybridization between ancestors of *Brassica oleracea* (CC, 2n = 18) and *Brassica rapa* (AA, 2n = 20) (U, 1935; Palmer et al., 1983; Song and Osborn, 1992; Allender and King, 2010). Natural euploid *B. napus* displays predominantly 19 bivalents at Metaphase I (MI) and an almost strict disomic inheritance; this shows that the vast majority of COs are formed between homologous chromosomes. Evidence for rare homoeologous exchanges was obtained in several *B. napus* cultivars (Parkin et al., 1995; Sharpe et al., 1995; Lombard and Delourme, 2001; Osborn et al., 2003; Piquemal et al., 2005; Udall et al., 2005; Howell et al., 2008), but their frequency remains very low compared with the rate in resynthesized *B. napus* (Parkin et al., 1995; Sharpe et al., 1995; Udall et al., 2005; Lukens et al., 2006; Gaeta et al., 2007; Szadkowski et al., 2010). Contrary to wheat, comparisons of meiosis among *B. napus* allohaploid plants, which carry one copy of each of the 10 A and 9 C *B. napus* chromosomes (AC), showed that allohaploids produced from some varieties displayed only a few univalents (i.e., chromosomes that fail to form a CO), whereas those produced from other varieties displayed mostly univalents (Olsson and Hagberg, 1955; Renard and Dosba, 1980; Attia and Röbbelen, 1986; Jenczewski et al., 2003). The quantitative trait loci (QTL) determining these phenotypes were mapped in a single population. A major locus (*PrBn*; Jenczewski et al., 2003) localized to linkage group C9, and four to six other additive or epistatic loci (Liu et al., 2006) were identified. Due to the polyphyletic origin of *B. napus*, it was logical to ask if *PrBn* is still the main determinant for CO suppression between homoeologs at the entire species level.

Our goal in this study was to decipher whether the diversity of meiotic behavior found among a wide range of *B. napus* allohaploid accessions is related to the polyphyletic origin of this species and diversity at the *PrBn* locus. We first characterized the meiotic behavior at MI in allohaploids produced from 29 *B. napus* varieties representing a range of genetic and geographic origins. We then analyzed chloroplast diversity in all these varieties to assess their maternal origins. Finally, we assayed molecular markers surrounding *PrBn* to reconstruct different multilocus genotypes for this region. Altogether, our results indicate that variation in CO frequency among allohaploid genotypes roughly correlates with the multiple origins of *B. napus* and *PrBn* diversity. More complex patterns of meiotic and genetic diversity were also observed. Our findings highlight the diverse nature of homoeologous recombination regulation in the wild.

RESULTS

Diversity of Meiotic Behavior among *B. napus* Allohaploids

A total of 363 allohaploid plants were isolated from 29 varieties representing different types of cultivars and a major part of *B. napus* genetic diversity (Table 1). We analyzed the meiotic behavior at MI of all allohaploids and one raw *B. oleracea* × *B. rapa* interspecific hybrid, which have a similar karyotype (10 A and 9 C chromosomes) (Figures 1 and 2; see Supplemental Data Set 1 online). Homologous chromosomes are absent in all these plants; thus, the chiasmata observed on bivalents and multivalents (Figure 1B) mark the sites of meiotic COs between homoeologous and/or nonhomologous chromosomes (Nicolas et al., 2007). We also observed varying numbers of univalents in all plants (Figure 1). We used the number of univalents to describe the MI meiotic behavior of *B. napus* allohaploids because this variable is directly correlated to the extent of CO formation between homoeologous or nonhomologous chromosomes (Nicolas et al., 2009). All the *B. napus* allohaploids displayed a significantly higher number of univalents than did the A×C interspecific hybrids (F-test; $P < 0.001$; Figure 2), suggesting that the number of COs between homoeologous/nonhomologous chromosomes was reduced in the *B. napus* background regardless of the accession.

We identified two clear-cut meiotic phenotypes among all *B. napus* allohaploids (Figures 1 and 2). Allohaploids isolated from a first group of 19 accessions, including *Darmor-bzh*, showed a high level of recombination between homoeologous/nonhomologous chromosomes; they had from 3.57 to 5.14 univalents per accession on average, mean numbers of univalents per allohaploid plant not higher than 6 (Figures 1A, 1B, and 2), and 9 to 12 chiasmata per pollen mother cell (PMC). We named this group fu, for few univalents. By contrast, allohaploids produced from a second group of seven accessions, including *Yudal*, showed a low level of recombination between homoeologous /nonhomologous chromosomes; they displayed from 8 to 11.4 univalents per accession on average, mean numbers of univalents per allohaploid plant not lower than 8 (Figures 1E, 1F, and 2), and two to six chiasmata per PMC. We called this group MU, for many

Table 1. Accessions Used to Assess and Compare Patterns of Meiotic, Plastid, and Nuclear Marker Diversity

Species	Accession Name	Country of Origin	Plant Breeder[a]	Pop. Type	Growth Habit	No. of Parental Plants	No. of Haploids	GenBank[a]	GenBank Accession No.
B. rapa	Z1	Canada	AAFC	Line				AAFC	
B. oleracea	HDEM	France	INRA	Line				INRA Rennes	
B. napus spp *oleifera*	Akamar	Holland	VDH	Population	Winter	4	25	INRA Rennes	
B. napus spp *oleifera*	Brutor	France	Ringot	Line	Spring	1	8	INRA Rennes	
B. napus spp *oleifera*	Capricorn	UK	PBI	Population	Winter	4	24	INRA Rennes	
B. napus spp *oleifera*	Drakkar	France	INRA-Serasem	Line	Spring	1	8	INRA Rennes	
B. napus spp *oleifera*	Darmor-bzh	France	INRA-Serasem	Line	Winter	1	59	INRA Rennes	
B. napus spp *oleifera*	Eurol	France	Cargill	Line	Winter	1	1	INRA Rennes	
B. napus spp *oleifera*	Garant	Germany	Lembkes	Population	Winter	3	9	INRA Rennes	
B. napus spp *oleifera*	Hinchu	Korea	Nokpo Univ.	Line	Spring	1	2	INRA Rennes	
B. napus spp *pabularia*	asparagus kale			Population	Spring	3	12	WGB	6224
B. napus spp *oleifera*	Jet Neuf	France	Ringot	Line	Winter	1	5	INRA Rennes	
B. napus spp *oleifera*	Loras	Germany	Petkus	Population	Spring	4	12	GGB	734
B. napus spp *oleifera*	Maluka	Australia	NSWDA	Line	Spring	1	1	INRA Rennes	
B. napus spp *oleifera*	Maxol	France	Cargill	Line	Winter	1	7	INRA Rennes	
B. napus spp *oleifera*	Mohican	UK	CPB	Population	Winter	4	33	INRA Rennes	
B. napus spp *oleifera*	Nachan	Korea	Nokpo Univ	Line	Spring	1	8	INRA Rennes	
B. napus spp *oleifera*	Norin 1	Japon	Fukuoka	Line	Spring	1	9	INRA Rennes	
B. napus spp *oleifera*	Norin 6	Japon	OOSaka	Line	Spring	1	10	INRA Rennes	
B. napus spp *oleifera*	Norin 9	Japon	OOSaka	Line	Spring	1	4	INRA Rennes	
B. napus spp *oleifera*	Norin 10	Japon	Fukushima	Line	Spring	1	9	INRA Rennes	
B. napus spp *oleifera*	Oro	Canada	AAFC	Population	Spring	5	13	AAFC	
B. napus spp *oleifera*	Petranova	Germany	Petkus	Line	Winter	1	3	INRA Rennes	
B. napus spp *rapifera*	Rutabaga 22	France	–	Line	Winter	1	8	INRA Rennes	
B. napus spp *rapifera*	Rutabaga 85	France	–	Line	Winter	1	10	INRA Rennes	
B. napus spp *oleifera*	Samouraï	France	INRA-Serasem	Line	Winter	1	5	INRA Rennes	
B. napus spp *oleifera*	Spok	Germany	Dansk Plants Foraedling	Line	Spring	1	7	INRA Rennes	
B. napus spp *oleifera*	Stellar	Canada	Univ. Manitoba	Line	Spring	1	8	INRA Rennes	
B. napus spp *oleifera*	Taichung	Korea	Nokpo Univ	Line	Spring	1	6	INRA Rennes	
B. napus spp *oleifera*	Westar	Canada	AAFC	Line	Spring	1	5	INRA Rennes	
B. napus spp *oleifera*	Yudal	Korea	Nokpo Univ	Line	Spring	1	51	INRA Rennes	

[a]AAFC, Agriculture and AgriFood Canada; INRA, Institut National de la Recherche Agronomique; VDH, van der Have BV; PBI, Plant Breeding International Cambridge; NSWDA, New South Wales Department of Agriculture; CPB, Cambridge Plant Breeder Twyford; WGB, Wellsbourne Gene Bank, Horticulture Research International; GGB, Gatersleben GeneBank, Institure of Plant Genetics and Crop Plant Research.

univalents. Similar differences in the number of multivalents were observed between the fu and MU groups: 128 trivalents (III) and 281 quadrivalents (IV) but only 31 III and 16 IV were scored for the fu and MU allohaploids, respectively. The remaining three accessions (*Loras*, *Oro*, and *Norin9*) produced a mixture of allohaploids with either the fu or MU meiotic phenotype (Figures 1C, 1D, and 2).

Only eight allohaploids showed an intermediate meiotic phenotype (with mean numbers of univalents between 6 and 8), six of which originated from *Loras*, *Oro*, and *Norin9* (Figure 2). A detailed analysis of the frequency of univalents per PMC revealed that four of them remained very similar to the MU or fu allohaploids, differing only in one additional/missing pair of univalents (e.g., *Norin9-02* and *Loras-07* in Supplemental Figure 1 online). The distribution of the number of univalents per PMC was clearly different for the remaining four intermediate allohaploids. Many of their MI cells had univalent scores that were rarely observed in MU/fu allohaploids (e.g., please compare *Oro-09* and *Loras-09* to *Darmor-bzh* and *Yudal* in Supplemental Figure 1 online). Thus, the meiotic phenotypes of only these four allohaploids (out of 363) did not noticeably match the clear-cut fu/MU dichotomy.

Slight differences were sometimes observed between allohaploids produced from a given accession (Figure 2). In general, statistically significant differences were found between allohaploids produced from every MU accession ($P = 0.007$; Wald *Z*-tests), whereas no significant difference was found between allohaploids isolated from fu accessions ($P = 0.0936$; Wald *Z*-tests). In some cases, the number of univalents determined for different allohaploids isolated from the same accession differed depending on their spatial locations in the greenhouse, suggesting a slight environmental effect across the experimental area (see Supplemental Figure 2 online). The response surfaces appeared to be the same between the different mother plants representing an open-pollinated population and between some accessions, but they were different between some others (see

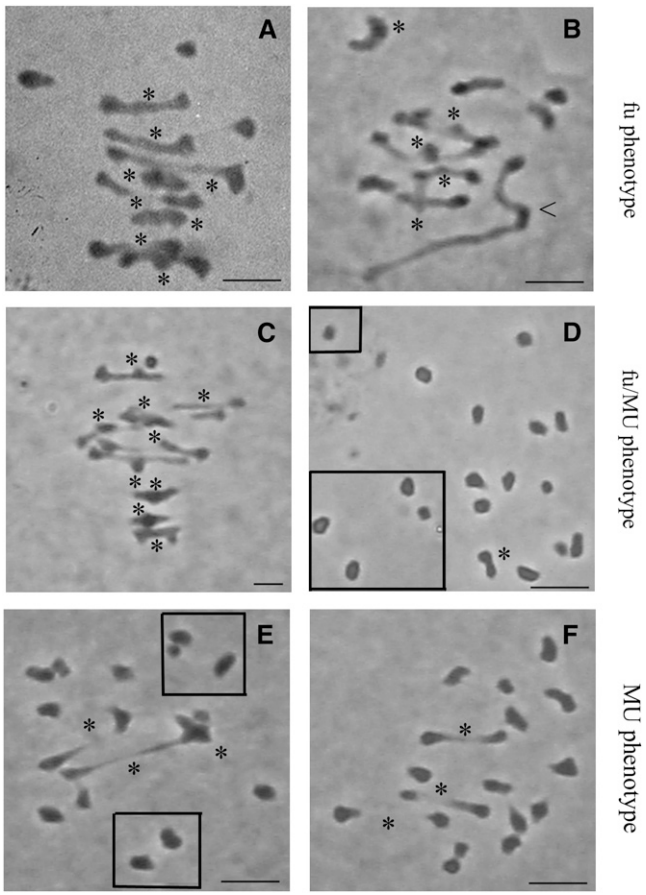


Figure 1. Representative MI Nuclei of *B. napus* Allohaploids Showing Contrasting Meiotic Behaviors.

(A) *Darmor-bzh*: eight bivalents (II) + three univalents (I).
(B) *Norin 1*: one quadrivalent, 5II +4I.
(C) and (D) *Norin 9*: 9II + 1I – 1II + 17I,
(E) *Garant*: 3II + 13I.
(F) *Yudal*: 3II + 13I.
The univalents located peripherally (out of the frame of these high-magnification micrographs) are indicated within squares (**[D]** and **[E]**). Bivalents are indicated with an asterisk, and the quadrivalent in **(B)** is indicated with an arrowhead. Bars = 5 μm.

Supplemental Figure 2 online). This meant that spatial autocorrelation could not be introduced into the statistical models. It is also apparent in Figure 2 that variance among allohaploids isolated from MU accessions increased when the mean increased, a relationship that was taken into account in subsequent statistical analyses.

Analyses of variance, performed separately on the fu and MU groups, showed that significant differences existed between accessions within each group ($P < 0.001$ for both analyses). As no significant difference was observed between the different mother plants representing an open-pollinated population (Figure 2), these data were pooled before we analyzed the extent to which the number of univalents varied between accessions. Significant block effects were also found for fu allohaploids ($P = 0.04$; Wald *Z*-tests), demonstrating that a small but significant fraction of spatial and temporal heterogeneity was captured while testing for differences between accessions.

Variation between accessions within the fu or MU groups was continuous, with pairwise differences divided into a series of overlapping subgroups. No clear-cut subgroup could thus be recognized. However, it was possible to identify accessions that were significantly different from *Darmor-bzh* and *Yudal*, the two varieties we used in previous analyses (Jenczewski et al., 2003; Liu et al., 2006; Nicolas et al., 2009). We found that the number of univalents in allohaploids originating from six accessions (*Akamar*, *Brutor*, *JetNeuf, Nachan*, *Norin10*, and *Taïchung*), plus the fu allohaploids produced from *Loras* and *Oro*, was slightly but significantly higher than in *Darmor-bzh* allohaploids (see Supplemental Table 1 online). Likewise, the single fu allohaploids isolated from *Maluka* and *Norin9* differed from *Darmor-bzh* allohaploids, although no statistical comparison was possible. By contrast, no *B. napus* allohaploid produced significantly fewer univalents than did *Darmor-bzh*. In the MU group, when variance stabilizing corrections were applied, only the allohaploids originating from *Hinchu* plus the MU allohaploids produced from *Loras* and *Oro* displayed significantly fewer univalents than did *Yudal* allohaploids (see Supplemental Table 1 online); only *Asparagus kale* produced significantly more univalents at the allohaploid stage than did *Yudal*.

Finally, we estimated that the variance for the number of univalents attributable to differences between the fu and MU groups (estimated at 18.54) was considerably larger than any other source of variation (for example, the variance attributable to differences between accessions from the same group was estimated at 0.3231). Due to the polyphyletic origin of *B. napus*, we addressed the hypothesis that these two main meiotic phenotypes could have originated from independent polyploidization events.

Diversity of Plastid Haplotypes among *B. napus* Accessions and Their Distribution with Respect to Allohaploid Meiotic Behavior

We first analyzed the genetic diversity of the chloroplast genome to gain insight into the maternal origins of the 29 *B. napus* accessions.

Sequencing both the *ndhC-trnV* and *rbcL-accD* chloroplast intergenic regions showed exactly the same extent of chloroplast diversity. Only two plastid genome (ptDNA) haplotypes were found; they differed by a total of nine single nucleotide polymorphisms and three indels (insertion/ deletion), including a 10-bp deletion (see Supplemental Data Set 2 online). Twenty four accessions, including the two rutabagas (*B. napus* ssp *rapifera*), had the same ptDNA haplotype, which was different from the ptDNA haplotypes found in the *B. rapa* and *B. oleracea* genotypes examined in this study (ptDNA haplotype 1; Figure 2; see Supplemental Data Set 2 online). The remaining five *B. napus* accessions, including asparagus kale (*B.napus* ssp *pabularia*), shared a second ptDNA haplotype that was more closely related to that of *B. rapa* Z1 and *B. oleracea* HDEM genotypes (ptDNA haplotype 2; Figure 2; see Supplemental Data Set 2 online). The plastid haplotypes of *B. napus* accessions did not appear to be

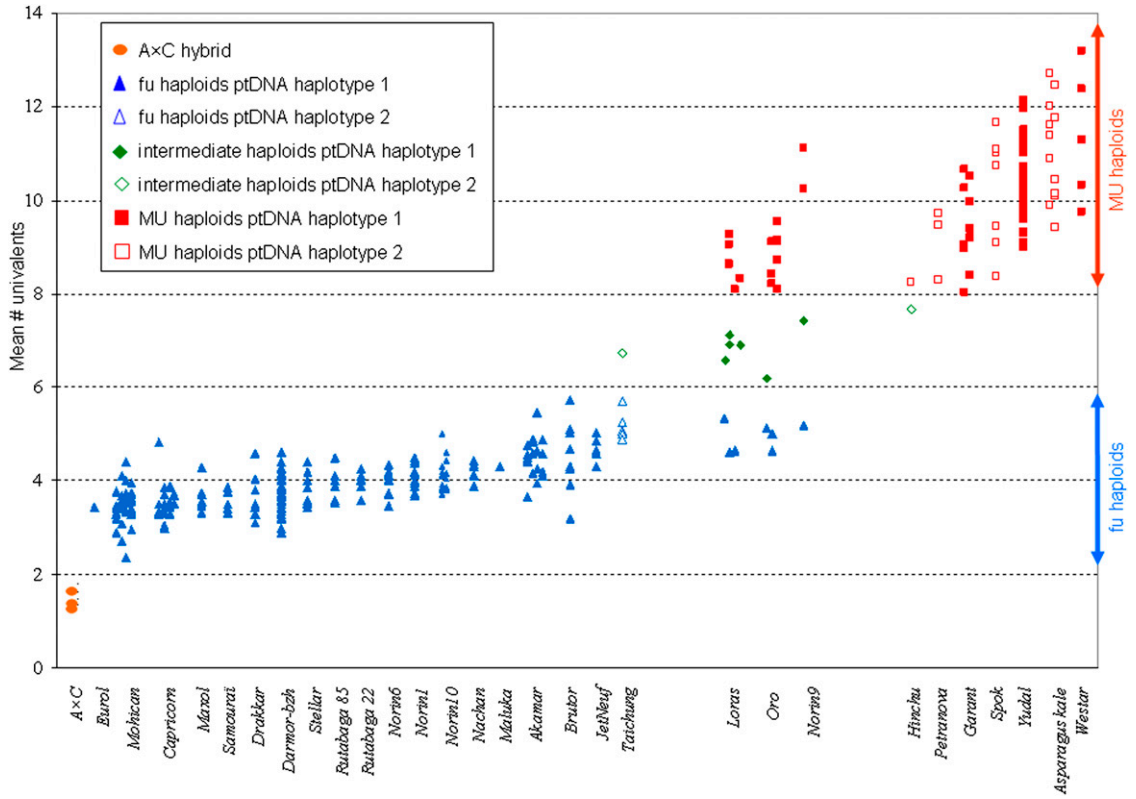


Figure 2. Diversity of Meiotic Behavior in *B. napus* Allohaploids.

Symbols represent the mean number of univalents (calculated for ~20 PMCs) for every allohaploid plant isolated from the 29 *B. napus* accessions listed on the *x* axis and for five interspecific *B. oleracea* × *B. rapa* hybrids (noted A×C). Symbols with the same X-coordinate represent allohaploids isolated from the same plant. The clusters of consecutive X-coordinate samples represent three to four distinct plants sampled from the same population to account for its potential genetic heterogeneity (e.g., *Mohican*, *Capricorn*, etc.). Triangles represent allohaploids showing a high level of homoeologous recombination (fu allohaploids), diamonds represent allohaploids with an intermediate meiotic behavior, and squares represent allohaploids that showed a low level of homoeologous recombination (MU allohaploids). The two plastid haplotypes are represented by open and closed symbols, respectively.

[See online article for color version of this figure.]

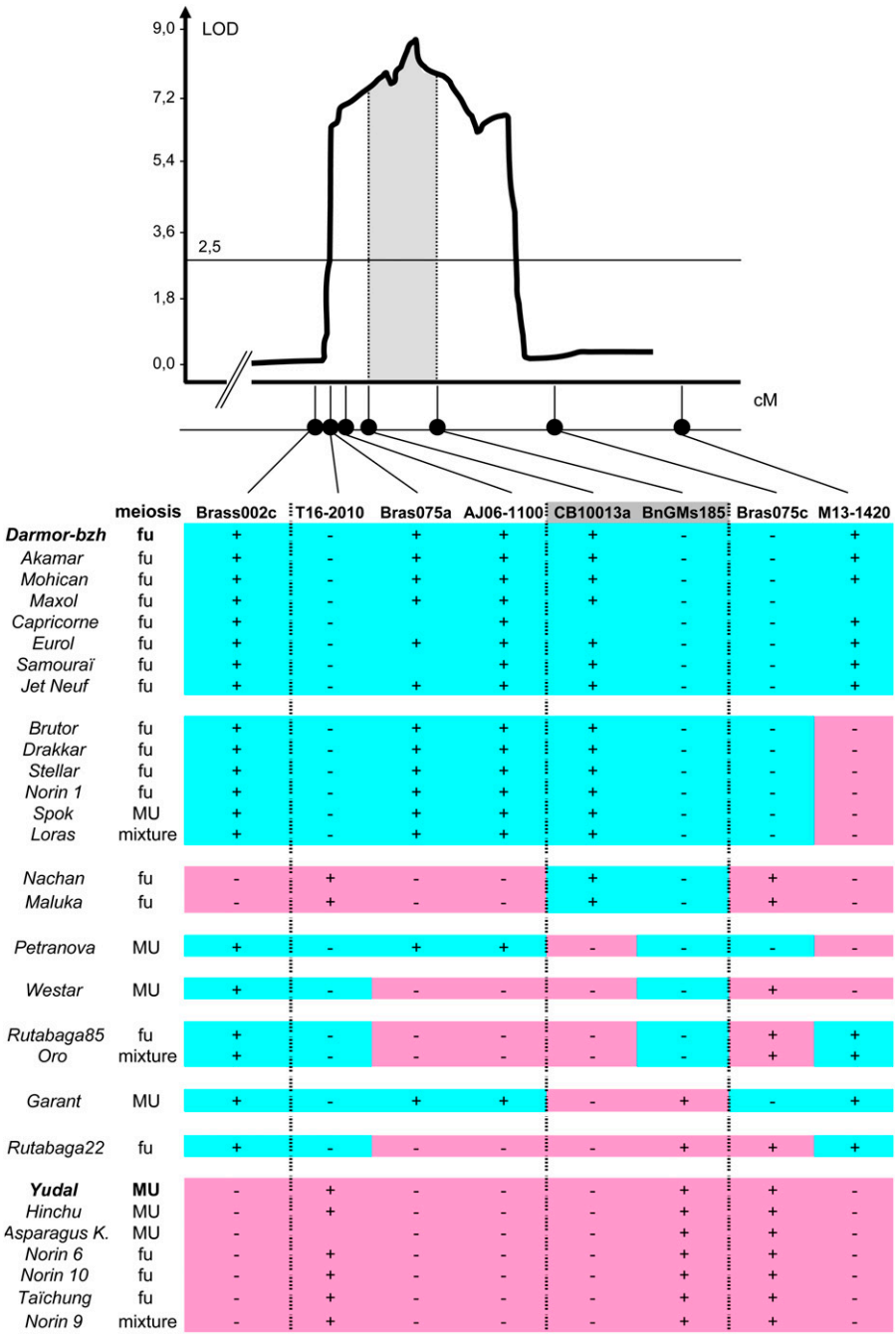
completely randomly distributed with regards to the geographic origin; in particular, ptDNA haplotype 2 is found in German and Korean (Figure 2, Table 1) oilseed accessions as well as in *B. napus* ssp *pabularia*, which is thought to come out of Northern Europe/Asia.

We then compared the patterns of meiotic and ptDNA diversity and observed that the two plastid haplotypes were not randomly distributed with respect to the two main meiotic behaviors (Fisher's exact test, P = 0.0123). Indeed, almost all the fu accessions (18 out of 19) displayed ptDNA haplotype 1, whereas a more balanced mix of ptDNA haplotypes was found among MU accessions (Figure 2). ptDNA haplotype 1 was present in *B. napus* accessions that produced a mixture of allohaploids with either the MU or fu meiotic phenotype (*Loras*, *Oro*, and *Norin9*) (Figure 2). Of note, the distribution of the two meiotic phenotypes appeared to be independent from any other source of clustering, such as the winter and spring oilseed types (Fisher's exact test, P > 0.05).

Overall, our results confirmed that *B. napus* arose at least twice and suggest that the dichotomy of meiotic behaviors among *B. napus* allohaploids could be related to these multiple origins. To gain further insight into the origin of the natural variability for homoeologous recombination in *B. napus*, we analyzed genetic diversity at the *PrBn* locus (Jenczewski et al., 2003; Liu et al., 2006).

Nuclear DNA Marker Diversity in the *PrBn* Region with Respect to the Allohaploid Meiotic Behavior

We analyzed the genetic diversity of eight molecular markers spanning a region of ~60 centimorgans (cM) centered on the peak of the QTL (between CB10013a and BnGMs185 markers) defined by Liu et al. (2006) as *PrBn* (Figure 3). Most of these markers were dominant so that our screening mainly resulted in binary band presence-absence patterns. We considered that bands showing the same electrophoretic mobility between different accessions were identical-by-descent and that all accessions that showed no band for a dominant marker had the same allele at that locus.



	meiosis	Brass002c	T16-2010	Bras075a	AJ06-1100	CB10013a	BnGMs185	Bras075c	M13-1420
Darmor-bzh	**fu**	+	-	+	+	+	-	-	+
Akamar	fu	+	-	+	+	+	-	-	+
Mohican	fu	+	-	+	+	+	-	-	+
Maxol	fu	+	-	+	+	+	-	-	
Capricorne	fu	+	-		+		-	-	+
Eurol	fu	+	-	+	+	+	-	-	+
Samouraï	fu	+	-		+	+	-	-	+
Jet Neuf	fu	+	-	+	+	+	-	-	+
Brutor	fu	+	-	+	+	+	-	-	-
Drakkar	fu	+	-	+	+	+	-	-	-
Stellar	fu	+	-	+	+	+	-	-	-
Norin 1	fu	+	-	+	+	+	-	-	-
Spok	MU	+	-	+	+	+	-	-	-
Loras	mixture	+	-	+	+	+	-	-	-
Nachan	fu	-	+	-	-	+	-	+	-
Maluka	fu	-	+	-	-	+	-	+	-
Petranova	MU	+	-	+	+	-	-	-	-
Westar	MU	+	-	-	-	-	-	+	-
Rutabaga85	fu	+	-	-	-	-	-	+	+
Oro	mixture	+	-	-	-	-	-	+	+
Garant	MU	+	-	+	+	-	+	-	+
Rutabaga22	fu	+	-	-	-	-	+	+	+
Yudal	**MU**	-	+	-	-	-	+	+	-
Hinchu	MU	-	+	-	-	-	+	+	-
Asparagus K.	MU	-		-	-	-	+	+	-
Norin 6	fu	-	+	-	-	-	+	+	-
Norin 10	fu	-	+	-	-	-	+	+	-
Taïchung	fu	-	+	-	-	-	+	+	-
Norin 9	mixture	-	+	-	-	-	+	+	-

Figure 3. Genotype of the Surveyed Accessions in the Region Surrounding *PrBn*, the Main Determinant for the Number of Univalents among *B. napus* Allohaploids.

The likelihood ratio profile at the top of the figure was modified from Liu et al. (2006); this profile was generated by composite interval mapping with a LOD score threshold of 3.3. *x* axis, map distances in cM; *y* axis, LOD score. Markers were ordered according to published genetic maps (Delourme et al., 2006, 2008; Liu et al., 2006). The contrasted genotypes of the *Darmor-bzh* and *Yudal* accessions, which were the parents of the segregating population used to map *PrBn* (Liu et al., 2006), were used to identify alleles for every marker: open cells represent *Darmor-bzh*-like alleles, while closed cells represent *Yudal*-like alleles. + Indicates that the band was present, and − indicates that the band was absent.
[See online article for color version of this figure.]

The most significant association with meiotic variation was observed for CB10013a ($\chi^2 = 6.86$; P = 0.008): 19 accessions (out of 25; 75%) had a CB10093a allele that matched their meiotic behavior and 37% of the overall variation for the number of univalents was accounted for by allele segregation at CB10013a. Owing to the fact that MU and fu meiotic phenotypes are both found across the most distinct genetic pools of *B. napus* (spring and winter oilseed types; see above), this association certainly reflects the close proximity of CB10093a to *PrBn* (Figure 3).

This conclusion is supported by our modeling of multilocus genotypes and their comparison to those of the *Darmor-bzh* and *Yudal* accessions, the two varieties used to identify and map *PrBn* (Jenczewski et al., 2003; Liu et al., 2006). We first identified a total of nine multilocus genotypes (Figure 3). The two most frequent multilocus genotypes were similar to those of *Darmor-bzh* and *Yudal*, respectively; seven accessions had the same multilocus genotype as *Darmor-bzh* (this number extends to 13 if marker M13-1420, which is located ~25 cM away from the peak of the QTL, is not considered), and six accessions shared the same multilocus genotype with *Yudal* (Figure 3). The remaining accessions had a recombinant multilocus genotype between these two archetypes, two of these showing a single marker data point that produced a double recombinant genotype. These two singletons were repeatedly found in reiterated experiments that included flanking markers.

We then compared the patterns of meiotic behavior and multilocus genotype diversity. MU accessions appeared more diverse (five multilocus genotypes out of seven accessions) than did fu accessions (six multilocus genotypes out of 19 accessions). However, the multilocus genotype in the *PrBn* region of most accessions matched their meiotic phenotype. Eleven fu accessions (out of 18) had the same multilocus genotype as *Darmor-bzh*, while two MU accessions (out of six) had the same multilocus genotype as *Yudal*. Focusing on the region near the peak of the *PrBn* QTL (between CB10013a and BnGMs185 markers) revealed that the profiles of all but one of the recombinant multilocus genotypes could match their MU/fu phenotype (see Supplemental Figure 3 online). Actually, the meiotic phenotype of only five accessions did not match their *PrBn* genotype; four accessions (*Norin6*, *Norin10*, *Taïchung*, and *Rutabaga 22*) had the same multilocus genotype as *Yudal* in the interval surrounding *PrBn* but behaved like *Darmor-bzh* allohaploids at MI; reciprocally, one accession (*Spok*) had the same multilocus genotype as *Darmor-bzh* at *PrBn* but behaved like *Yudal* allohaploids at MI (Figure 3). The three accessions that gave a mixture of fu or MU allohaploids displayed three unrelated multilocus genotypes (Figure 3): one similar to that of *Darmor-bzh* (*Loras*), one similar to that of *Yudal* (*Norin9*), and one recombinant multilocus genotype (*Oro*).

DISCUSSION

Natural and resynthesized *B. napus* are known to display very different levels of meiosis regularity and genome stability (for review, see Gaeta and Pires, 2010) that are reflected here by the reduction in CO between homoeologs in *B. napus* allohaploids compared with raw *B. oleracea* × *B. rapa* interspecific hybrids (Figure 2). In principle, this difference could reflect chromosome rearrangements that accentuated the divergence between *B. napus* homoeologous chromosomes after the inception of this species. However, although a few genetic changes were detected in several *B. napus* cultivars (references cited in the introduction), accumulating evidence indicates that the *B. napus* A/C genomes remain essentially the same as the A/C genomes of their progenitors (Bohuon et al., 1996; Parkin and Lydiate, 1997; Rana et al., 2004; Suwabe et al., 2008; Cheung et al., 2009). This suggests that natural *B. napus* has evolved or inherited *Pairing-homoeologous* loci that ensure proper chromosome recombination and segregation (such as *PrBn*; Jenczewski et al., 2003; Liu et al., 2006; Nicolas et al., 2009).

Our study showed that this genetic regulation occurs with varying degrees of stringency, at least among *B. napus* allohaploids. We effectively observed natural variation for recombination between homoeologous chromosomes that relied on two clear-cut meiotic phenotypes. Rough estimates of chiasma frequencies indicated that MU allohaploids displayed a range of two to six chiasmata per PMC, whereas the number of chiasmata per PMC varied from 9 to 12 in fu allohaploids (see also Renard and Dosba, 1980; Attia and Röbbelen, 1986). As already stated in Nicolas et al. (2009), this variation does not reflect a difference in the number of chiasmata that are formed on the recombining bivalents, which are strikingly similar between fu and MU allohaploids (~1.4 COs per bivalent on average). Conversely, it results from a difference in the number of chromosomes that form bivalents or multivalents during meiosis (Nicolas et al., 2009). In *B. napus* allohaploids, bivalents are not always formed between homoeologous chromosomes, as autosyndetic pairs (A-A or C-C) were also observed in the same proportion at meiosis of *Darmor-bzh* fu and *Yudal* MU allohaploids (Nicolas et al., 2007, 2009). Because these two genotypes are representative of the whole range of meiotic phenotypes (Figure 2), we would not expect more than slight variations in the distribution of COs between homoeologous and other nonhomologous chromosomes among *B. napus* allohaploids.

Our worldwide sample encompassed three main *B. napus* gene pools (ssp *oleifera*, ssp *rapifera*, and ssp *pabularia*), included both winter and spring oilseed types (Diers and Osborn, 1994; Lombard et al., 2000; Hasan et al., 2006), and thus represented a major part of *B. napus* genetic diversity. Although phenotyping other *B. napus* accessions could provide supplementary information, the dichotomy of meiotic behaviors found here certainly highlights the overall variation present in this species, which contains germplasm of shared recent common ancestry (Prakash and Hinata, 1980; Allender and King, 2010). This observation and the fact that no other meiotic behavior was found in a handful of other accessions (Olsson and Hagberg, 1955; Renard and Dosba, 1980; Attia and Röbbelen, 1986; Tai and Ikonen, 1988) suggest that most *B. napus* allohaploids display either a high (MU phenotype) or a low (fu phenotype) number of univalents, with only slight variations within these two canonical phenotypes (Figure 2). These slight variations may reflect either the segregation of genes modifying chiasma frequency or, alternatively, the occurrence of chromosomal changes that differ between accessions (discussed in Udall et al., 2005; Liu et al., 2006).

Only four allohaploids (out of 363) showed a meiotic behavior between the MU and fu phenotypes (Figure 2). Three of these originated from accessions that also produced a mixture of fu and MU allohaploids (*Oro* and *Loras*). These results are reminiscent of those obtained in the segregating population of allohaploids isolated from *Darmor-bzh* × *Yudal* F1 hybrids (Jenczewski et al., 2003). Thus, it is possible that *Oro* and *Loras* are heterozygous for some determinants of CO formation between homoeologous chromosomes. *Loras* and *Oro* were obtained by open pollination and, therefore, potentially contain a bulk of more or less related genotypes. However, the explanation is not straightforward for *Norin9*, which was thought to be a homogeneous line but produced a mixture of fu and MU allohaploids.

The clear-cut dichotomy of meiotic phenotypes found among *B. napus* allohaploids is in contrast with the slight differences observed between wheat allohaploids (*T. aestivum*; ABD $n = 21$; Martinez et al., 2005 and references therein) and tall fescue (*Festuca arundinacea*; ABC $n = 21$; Eizenga and Kasperbauer, 1985) in which the number of univalents varied only from 16 to 20. Although this narrow range in wheat could be due to the small number of genotypes analyzed (three cultivars), whether it is a consequence of the polyploid history of wheat is yet to be determined. *Ph1* is the main determinant of CO suppression between homoeologs (Riley and Chapman, 1958). All polyploid wheat species, including *Triticum timopheevi* and *Triticum araraticum* (AAGG), show *Ph1* activity and ostensibly the same structure at the *Ph1* locus (Griffiths et al., 2006). Thus, the variation in CO suppression between homoeologs observed either between *T. aestivum* allohaploids (Martinez et al., 2005) or between tetraploid wheats × *Aegilops peregrina* hybrids (Ozkan and Feldman, 2001) remains unexplained.

Compared with wheat, our study provided more straightforward support for the hypothesis that repeated polyploidy has driven extensive variation in the determinants of CO suppression between homoeologous chromosomes in *B. napus*. Indeed, our results suggest that MU and fu allohaploids could originate from different maternal origins. All but one fu accession showed haplotype 1 ptDNA, and the almost perfect correlation between ptDNA haplotype and MU/fu phenotype (Figures 2) was disrupted only for four accessions (out of the 26 that did not produce a mixture of MU/fu allohaploids). These exceptions were expected due to the history of oilseed rape breeding, which involved a series of crosses between established cultivars (Salisbury and Wratten, 1999; Handa, 2007). For example, only two founder spring varieties were used to improve oilseed quality in the overwhelming majority of modern spring and winter oilseed genotypes (Hasan et al., 2008). Although the pedigrees of most accessions analyzed in this study are largely unknown, there is no reason to believe that these varieties were not the products of (extensive) intercrossing (see Handa, 2007 for the genealogy of Japanese varieties), which provided many opportunities for cytoplasm exchanges. Indeed, we observed that the high diversity of ptDNA found in the MU group (Figure 2) correlated with a high diversity of multilocus genotypes around *PrBn* (five out of seven accessions). These results are in close agreement with the findings of Sharpe and Lydiate (2003), who showed that the genome of oilseed rape cultivars is a mosaic of blocks from ancestral genotypes. We thus speculate that the MU and fu groups originated as genetically distinct lineages produced from at least two maternal diploid progenitors. At present, we do not know if the determinant conferring variation in meiotic behavior was directly inherited from alleles segregating in diploid progenitors or if it arose through subsequent introgressions or other genetic/epigenetic changes that independently accumulated in the different nascent lineages. Regardless of the original source of variation, subsequent crossing between accessions produced new combinations of meiotic and plastid types and the original evolutionary signal has faded.

B. napus ptDNA diversity definitely appears to have originated from independent polyploidization events. As *B. napus* is of very recent origin, it is very unlikely that the chloroplast of this species has accumulated such a high level of polymorphisms (nine single nucleotide polymorphisms and three indels; see Supplemental Data Set 2 online) since its inception. *B. napus* ptDNA diversity is also unlikely to have originated from a cytoplasm introgression from related species, which could have potentially occurred from *B.rapa* into some Asian *B. napus* accessions (Handa, 2007). All *Norin* accessions shared ptDNA haplotype 1, which was common to most other accessions but different from the ptDNA haplotype of the *B. rapa* genotype here. As proposed by Palmer et al. (1983), Erickson et al. (1983), and Song and Osborn (1992), ptDNA diversity provides evidence for at least two separate origins of *B. napus* involving two different maternal parents. However, Song and Osborn (1992) identified four maternal lineages instead of two, with most accessions showing the same plastid DNA as *Brassica montana*. Contrary to Song and Osborn (1992), we found that *Brutor* had the same ptDNA haplotype as almost all other accessions (Figure 2; see Supplemental Data Set 2 online). We analyzed different plastid regions than did Song and Osborn (1992), so this difference could reflect the idiosyncrasy of the *B. montana* chloroplast genome, which has local similarities with the other *B. napus* ptDNAs (Song and Osborn, 1992). Second, none of the accessions in our study had a *B. oleracea* ptDNA haplotype, which was represented only by the *New Zealand Rawara* accession in the Song and Osborn study; however, we found that this accession had only 18 chromosomes, suggesting that it belongs to the *B. oleracea* and not the *B. napus* germplasm (confirmed in Allender and King, 2010). Apart from these two points, our results confirmed the conclusions of Song and Osborn (1992). The two *B. napus* ptDNA haplotypes occurred in very different frequencies. Most accessions had ptDNA haplotype 1, and only five accessions had ptDNA haplotype 2 (Figure 2). We also confirmed that rutabagas had the main ptDNA haplotype (ptDNA haplotype 1), whereas asparagus kale had ptDNA haplotype 2. These results are also in agreement with the recent work of Allender and King (2010).

Finally, analysis of nuclear marker diversity in the *PrBn* region gave further support to the hypothesis that the dichotomy of meiotic phenotypes originated from (at least) two polyploidization events. The meiotic phenotype of 22 accessions (out of 29) was consistent with their multilocus genotype in the region surrounding *PrBn* (Figure 3). Half of these displayed the same multilocus genotype as did *Darmor-bzh* or *Yudal*, while the other half was recombinant in the interval with alleles at the peak of the QTL being in agreement with the meiotic phenotype (see Supplemental Figure 3 online). These results confirm the most likely

position for *PrBn* (between markers CB10013a and BnGMs185; Figure 3); owing to the rapid decay of linkage disequilibrium with distance in *B. napus* (Ecke et al., 2010), the statistical associations we found certainly reflects the close proximity of *PrBn* to the markers we used. These results also demonstrate that natural variation in meiotic behavior among *B. napus* allohaploids is consistent with the segregation of two alleles at the *PrBn* locus. This is the expected allelic composition if *B. napus* resulted from a double origin.

The meiotic phenotype of only eight accessions did not match their genotype in the region surrounding *PrBn* (Figure 3). Three of these were a mixture of MU/fu allohaploids, while they had the multilocus genotype fully or partly similar to that of *Darmor-bzh* or *Yudal*. These results echo the existence of similar allohaploids in the segregating population analyzed by Liu et al. (2006). Our study thus confirms that some accessions may have or segregate for different loci other than *PrBn*, which would modify the chance for homoeologous chromosomes to recombine. Although the origin, location, and mode of action of these loci remains to be determined (see Liu et al., (2006), our study provides a way of deliberately choosing genotypes to advance our understanding of the overall genetic architecture of the regulation of CO formation between homoeologous chromosomes in *B. napus*.

We showed that repeated polyploidy in *B. napus* probably resulted in different levels of CO suppression between homoeologs in allohaploids. We also confirmed that *PrBn* is still the major determinant for this trait at the species level. Interestingly Radoev et al. (2008) recently mapped a QTL that colocalizes with *PrBn* and is involved in determining the number of seeds per silique in a cultivar × resynthesized segregating population. As the number of seeds per silique is directly related to meiotic regularity, which is different between natural and resynthesized *B. napus* (for review, see Nicolas et al., 2008), this result could indicate a broader role for *PrBn* in regulating the diploid-like meiotic behavior of *B. napus*. What remains unclear then is the reason why all *B. napus* accessions display a diploid-like meiotic behavior, regardless of their genotype at the *PrBn* locus. Likewise a conventional cytological approach does not reveal any obvious difference of chiasmata between allotetraploids (see Supplemental Figure 4 online). However, this method is not sensitive enough to decipher if allohaploids with higher chiasma frequencies have mostly originated from allotetraploid accessions showing the higher values in this parameter. All these questions must be properly addressed before we can understand if "the tape of (meiosis) evolution would replay in the very same or similar way each time at the level of independently formed (*B. napus*) polyploid lines" (Soltis et al., 2009).

METHODS

Plant Material

Twenty-nine *Brassica napus* (AACC; 2n = 38) accessions were selected to represent broadly the genetic diversity present in this crop species (Table 1). Our selection encompassed the range of morphological forms that occur in this species and that are commonly used to divide it into three groups or subspecies: the oilseed crop *B. napus* ssp *oleifera* (represented by 26 accessions here), the Rutabaga *B. napus* ssp *napobrassica* or *rapifera* (represented by two accessions here), and *B. napus* ssp *pabularis* or *pabularia* (one accession: the asparagus kale). Molecular analyses confirmed that these three groups represent distinct gene pools (Song et al., 1988; Diers and Osborn, 1994). Our selection of oilseed accessions included both winter and spring types, which were shown to be differentiated gene pools (Diers and Osborn, 1994; Lombard et al., 2000; Hasan et al., 2006) and accessions from broad geographic origins, which was shown to correlate with the patterns of genetic diversity in this germplasm (Diers and Osborn, 1994; Chen et al., 2008). Most of the cultivars (22 out of 29) were represented by a single genetically homogeneous line, which was obtained after several generations of selfing (Table 1). A few cultivars (7 out of 29) were generated following open-pollination and therefore potentially contained a bulk of more or less related genotypes (so-called populations in Table 1). Finally three interspecific hybrids, which were all derived from a cross between *Brassica oleracea* cv *HDEM* and *B. rapa* cv *Z1*, were used for comparisons with *B. napus* allohaploids.

All allohaploids were isolated using microspore culture as described by Polsoni et al. (1988). Allohaploids were produced from a single plant when the cultivar was considered a genetically homogeneous line. When considering a population, allohaploids were produced separately from three to four distinct plants to account for potential genetic heterogeneity of the determinant influencing homoeologous recombination. *B. napus* accessions responded very differently to allohaploid production, so different numbers of allohaploids were generated for every genotype (Table 1). For some accessions, it even proved impossible to produce allohaploids through microspore culture. Thus, the number of accessions used in this study was smaller than an original larger selection (data not shown).

A total of seven microspore cultures were needed to isolate the 363 allohaploids analyzed here. Due to flowering time differences, eight series of allohaploids were successively grown in the greenhouse. For each series, allohaploid plants were randomly arranged on a grid in the greenhouse. Allohaploids produced from the two control accessions (*Darmor-bzh* and *Yudal*) were systematically and regularly dispersed across the entire grid. The *Darmor-bzh* and *Yudal* genotypes were the parents of the segregating allohaploid population that was used to map loci suppressing COs between homoeologous chromosomes (Jenczewski et al., 2003; Liu et al., 2006). Floral buds were sampled on the same date for all allohaploids of a series.

Meiotic Observations

Floral buds were fixed in Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1) for 24 h and stored in 50% ethanol. Observations on the PMCs were performed at the MI stage from anthers squashed and stained in a drop of 1% acetocarmine solution. On average, 20 PMCs were examined for each allohaploid.

Statistical Analysis

Statistical analyses were performed on the number of univalents because this variable was scored most reliably and measured the extent of homoeologous recombination in a synthetic way, reflecting by subtraction the number of chromosomes associated both as bivalents and multivalents. The model employed for each group was $Y_{glij} = \mu_g + \gamma_l + b_{g,i} + \varepsilon_{g,lij}$, where μ_g is the mean for group g (g is either group fu or MU), γ_l is the effect of accession l, $b_{g,i}$ is the random effect of block i, and $\varepsilon_{g,lij}$ is a residual error term. The $b_{g,i}$ and $\varepsilon_{g,lij}$ random effects were assumed to follow independent normal and centered distributions. In this design, each block included plants that were grown at the same time in two contiguous rows in the greenhouse; different blocks thus refer to either allohaploids from the same series but grown on rows separated by a walkway or allohaploids from different series that were grown at different

times in the greenhouse. The random block effect, which was estimated using the plants that were dispersed throughout each experimental area and in the different experiments, was used to account roughly for uncontrolled environmental variation (either across the area of each experiment or between experiments that followed one another in time). More sophisticated methods to account for prospective spatial autocorrelations were made inappropriate by the accession-dependant response surfaces (see Supplemental Figure 2 online). When required (notably for the MU group), heteroscedasticity was accommodated when running the model. All analyses were performed using the PROC MIXED procedure of SAS (SAS Institute, 1999). The PROC VARCOMP procedure of SAS (SAS Institute, 1999) was used to quantify and compare the effect of different factors of the models on the variability observed between allohaploids.

Amplification and Sequencing of Plastid Intergenic Regions

Intergenic regions *ndhC-trnV* and *rbcL-accD* from *Brassica* chloroplast were directly sequenced following PCR amplification (primer details are given in Supplemental Data Set 1 online). The thermal cycling profile was 35 cycles, each with 30 s denaturation at 94°C, 30 s annealing at 53°C, and an extension of 1 min at 72°C. A final extension of 10 min at 72°C was included. PCR products were sequenced by Genoscreen (France). Multiple sequence alignments were performed using default parameters of ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Molecular Analysis of the Region Surrounding *PrBn*

Eight molecular markers (see details in Supplemental Data Set 1 online) were selected from published genetic maps (Delourme et al., 2006, 2008; Liu et al., 2006; Cheng et al., 2009) to span the region within which *PrBn* was mapped as a QTL (Liu et al., 2006). We confirmed the position of GMs185 (Cheng et al., 2009) within this interval by selective genetic mapping, as detailed in Supplemental Data Set 1 online. PCR and electrophoresis were performed using the same protocols as described by Foisset et al. (1996) and Delourme et al. (2006).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Description of *B. napus* Allohaploids Showing an Intermediate Meiotic Behavior.

Supplemental Figure 2. Spatial Variation of Meiotic Behavior Measured for Allohaploids Isolated from the Same Plants but Positioned at Different Locations in the Greenhouse.

Supplemental Figure 3. Hypothetical Position of *PrBn* Locus Owing to the Multilocus Genotypes of Recombinant Varieties at the *PrBn* Region.

Supplemental Figure 4. Representative Metaphase I Nuclei of *B. napus* Allotetraploid Accessions Showing No Difference in Chiasma Frequency.

Supplemental Table 1. Differences of the LS Means between Accessions within the MU and fu Groups.

Supplemental Data Set 1. Markers and Averaged Meiotic Behaviors of All *B. napus* Allohaploids Used in This Study.

Supplemental Data Set 2. Multiple Alignments of *ndhC-trnV* and *rbcL-accD* Chloroplast Intergenic Sequences.

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