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Integration of the Cytogenetic and Genetic Linkage Maps of *Brassica oleracea*

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Manuscript received November 15, 2001
Accepted for publication March 25, 2002

ABSTRACT

We have assigned all nine linkage groups of a *Brassica oleracea* genetic map to each of the nine chromosomes of the karyotype derived from mitotic metaphase spreads of the *B. oleracea* var. *alboglabra* line A12DHd using FISH. The majority of probes were BACs, with A12DHd DNA inserts, which give clear, reliable FISH signals. We have added nine markers to the existing integrated linkage map, distributed over six linkage groups. BACs were definitively assigned to linkage map positions through development of locus-specific PCR assays. Integration of the cytogenetic and genetic linkage maps was achieved with 22 probes representing 19 loci. Four chromosomes (2, 4, 7, and 9) are in the same orientation as their respective linkage groups (O4, O7, O8, and O6) whereas four chromosomes (1, 3, 5, and 8) and linkage groups (O3, O9, O2, and O1) are in the opposite orientation. The remaining chromosome (6) is probably in the opposite orientation. The cytogenetic map is an important resource for locating probes with unknown genetic map positions and is also being used to analyze the relationships between genetic and cytogenetic maps.

THE genus Brassica (Cruciferae; Brassicae) is a major crop taxon of worldwide importance and is characterized by three basic diploid cytodemes (A, B, and C; $n = 10$, 8, and 9, respectively) and amphidiploid tetraploid species. The species *Brassica oleracea* (CC, $2n = 18$) includes the vegetable crops cauliflower, cabbage, calabrese, and Brussels sprouts. The amphidiploid *B. napus* (AACC, $2n = 38$) includes oil seed rape (Canola) and Swedish turnip. The genus is closely related to the model dicotyledonous plant *Arabidopsis thaliana* (Cruciferae; Sisymbrieae) for which the complete genome sequence has been established recently (Arabidopsis Genome Initiative 2000).

A cytogenetic map of Brassica associated with a genetic map will contribute to a wide range of research. It can provide information complementary to that from physical molecular maps currently being developed, as well as the location of genes in relation to features of chromosomal organization.

The integration of cytogenetic and genetic maps has been achieved for species such as wheat, barley, and *A. thaliana* utilizing chromosome deletions, translocation breakpoints, or trisomics (Koornneef and Vanderveen 1983; Künzel *et al.* 2000; Sandhu *et al.* 2001). However, these techniques can be impractical for some species due to difficulties in developing suitable lines and the absence of distinct cytological features. For potato (Dong *et al.* 2000) and *Medicago truncatula* (Kulikova *et al.* 2001), for example, fluorescence *in situ* hybridization (FISH) has proved to be an alternative approach. FISH probes range from large genomic DNA inserts in yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and cosmids to small genomic or cDNA inserts in plasmids. As chromosome markers, BACs have advantages over small single-copy probes because the signals they produce on metaphase chromosomes are easily recognized and are more consistently obtained (Hanson *et al.* 1995). Nonspecific signals caused by the hybridization of repetitive sequences, which are often present in large DNA fragments, can be suppressed with C_0t-1 DNA, thereby increasing the number of BACs that can be used successfully (Zwick *et al.* 1997; Dong *et al.* 2000).

Within the karyotype of *B. oleracea*, the definitive identification of all individual chromosomes within a mitotic metaphase spread can be problematic. This is primarily due to the small size of the chromosomes as well as similar chromosome lengths and/or arm ratios for some of the complement. The FISH technique has been used to assess the number and chromosomal position of 45S rDNA loci (*e.g.*, Snowdon *et al.* 1997; Hasterok *et al.* 2001) and 5S rDNA loci (Hasterok *et al.* 2001) and has aided the identification of 2 chromosomes with 45S rDNA loci in two karyotype studies of *B. oleracea* (Cheng *et al.* 1995; Fukui *et al.* 1998). In haploid lines of two *B. napus* cultivars, 10 *rapa*-type and 9 *oleracea*-type chromosomes have been characterized by their condensation pattern profiles. With FISH, using two cDNA probes in addition to 45S rDNA and 5S rDNA probes, five

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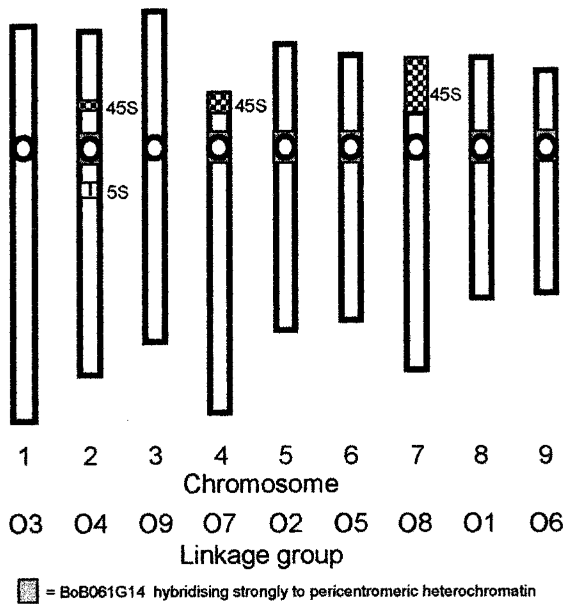

Figure 1.—Haploid idiogram of the karyotype of *B. oleracea* var. *alboglabra* line A12DHd.

chromosomes could be distinguished within the *oleracea*-type group. These chromosomes were not associated with linkage groups, although reference was made to the positions of 45S rDNA and the cDNA probes on genetic maps of *B. oleracea* and *B. napus* from other researchers (Kamisugi *et al.* 1998). A karyotype has been produced for *B. oleracea* var. *alboglabra* using chromosome size, arm ratios, and three repetitive DNA sequences as FISH probes. Five of the nine chromosomes of the haploid set (Figure 1) could be identified with more confidence using FISH than by using size parameters alone (Armstrong *et al.* 1998) but no links were made to a genetic map.

We describe a strategy that has allowed the assignment of all nine linkage groups to the nine chromosomes of the Brassica C genome. In this investigation we have used *B. oleracea* var. *alboglabra* doubled haploid line A12DHd (Bohuon *et al.* 1996), which has been used previously for the production of a partial karyotype (Armstrong *et al.* 1998) and a BAC library (C. D. Ryder and G. J. King, unpublished results; http://hbz.tamu.edu/bacindex4.html). It is also a parent of a doubled haploid line mapping population (Bohuon *et al.* 1996), the genetic map of which (A12DHdxGDDH33) has been integrated with another genetic map of *B. oleracea* (NxG; Sebastian *et al.* 2000). Probes, usually BACs, containing Brassica DNA sequences that have been genetically mapped to one or both of the mapping populations have been applied to mitotic and meiotic chromosomes from anthers. Confirmation that a BAC contains the mapped sequence has been achieved by one or more of the following methods: PCR, hybridization, comparison of restriction enzyme digestion patterns, and sequence analysis. Using the probes in various combinations, we have been able to associate each of the nine chromosomes with a particular linkage group. Since there is more than one probe for eight chromosomes, these chromosomes can be oriented with respect to the linkage group. We are using this cytogenetic map to locate other probes that cannot readily be mapped by segregation in these populations and to study the relationship between genetic and cytogenetic map positions.

MATERIALS AND METHODS

Genetic map markers: Twenty-three markers from a genetic map were selected for the purpose of linking the genetic map to the cytogenetic map. Each of the nine linkage groups was represented by at least two markers. Map positions of the 18 markers that were successfully assigned are shown in Figure 2.

Ten of the 23 markers, pW116E1, pW116J1, pW148E1, pW148E2, pO152J1, pO152E2, pN2J1, pR6E1, pR97J1, and pC14E1, were included in the integrated map of *B. oleracea* (Sebastian *et al.* 2000; http://www.biology.bham.ac.uk/brassica_map/). These were scored as restriction fragment length polymorphism (RFLP) markers using Brassica genomic plasmid clones pW148 and pW116 from the laboratory of T. Osborn (Madison, WI) and plasmid clones pO152, pN2, pR6, pR97, and cosmid clone C14 from the laboratory of I. Parkin (AAFC; Saskatoon, Saskatchewan, Canada). The RFLP autoradiographs for each of the plasmids had bands corresponding to the one or two mapped markers listed above but no monomorphic bands, indicating that the plasmid insert sequence was not present at other loci in the genome. Plasmid clones with an insert size >1.5 kb were selected because these were more likely than clones with smaller inserts to be detected with FISH.

Nine previously unreported markers were incorporated into the integrated map (Table 1 and Figure 2). Of these, eight were RFLP markers and one, HRI05, was a single nucleotide polymorphism (SNP) marker. RFLP filters were prepared and hybridized as described by Sebastian *et al.* (2000) using the following as RFLP probes: (1) a cDNA plasmid clone, EMBL J02798, kindly supplied by Lars-Gören Josefsson, Swedish University of Agricultural Sciences, Uppsala, for marker pNapin36; (2) a PCR product from BAC BoB039C15 using primers A and B (Brace *et al.* 1993) for marker BoSLGC; (3) a 383-bp PCR product of *BoAP1*-b, generated from A12DHd genomic DNA using the primers Fwd:CCAAGTATTTATAGTAGAC and Rev:ATACACATGCATGATATAAC with an annealing temperature of 50°; (4) a PCR product generated from A12DHd genomic DNA using primers based on EMBL X60324 for marker Bo45SIGS, which were Fwd:CAGCCCTTTGTCGCT AAG (within 25S gene) and Rev:GGCAGGATCAACCAGGTA (within 18S gene) with an annealing temperature of 61°; (5) locus-specific PCR products amplified from cDNA clones for markers HRI01, HRI02, and HRI03; and (6) a gene family probe, amplified from genomic Brassica DNA, for marker HRI04.

Four markers—for *BoAP1*-c (Smith 1999), *BoRGL*-IIIa, *BoRGL*-Iva, and *BoRGL*-Va (Vicente and King 2001)—were obtained through cleaved amplified polymorphic sequence (CAPS) assays.

Data were generated for the nine new markers from doubled haploid lines of the two mapping populations used in the production of the integrated map. Where a marker was monomorphic between the parents of one population, lines from this population were omitted. The segregation data for the additional markers were used to calculate map positions within the context of the existing integrated linkage map

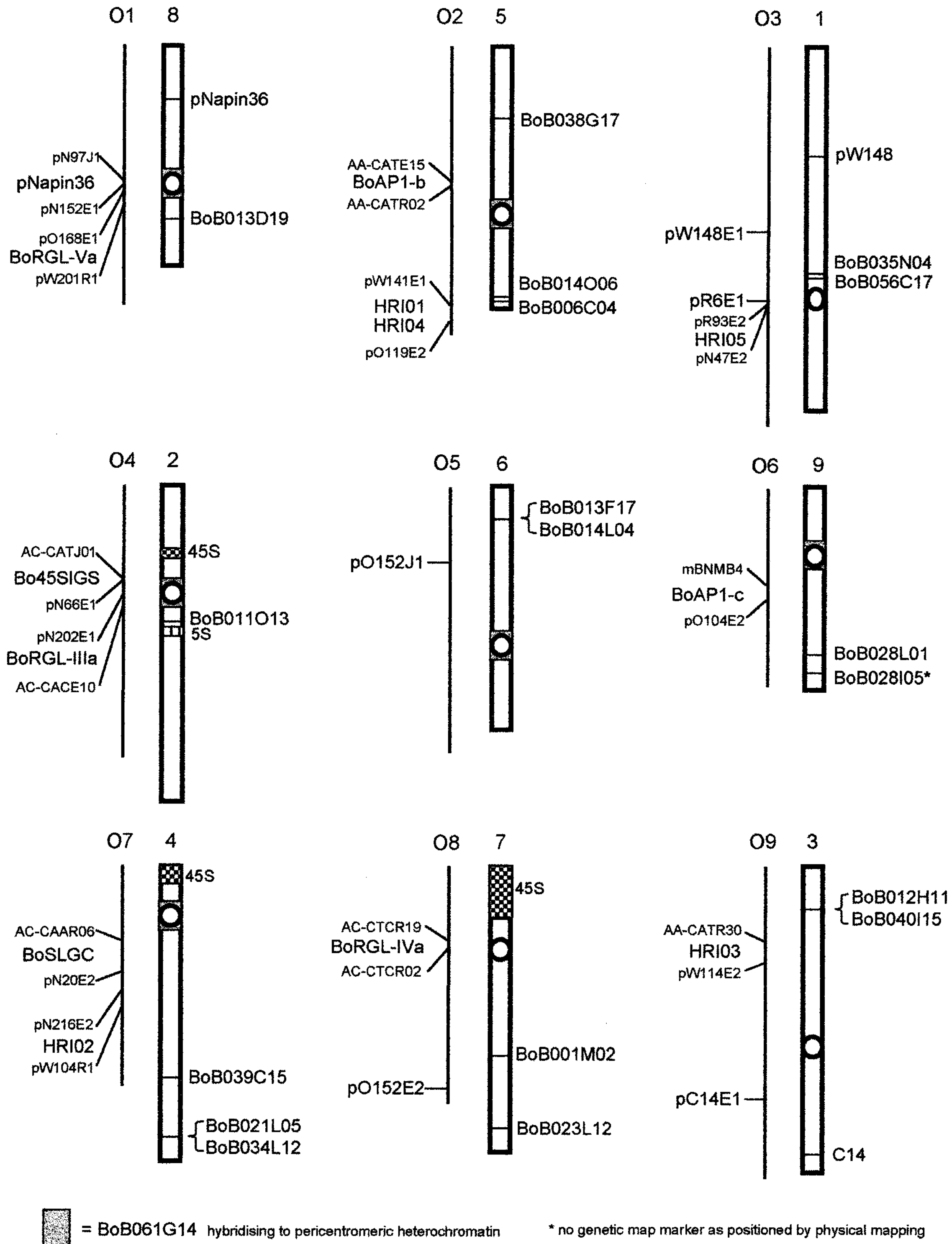

FIGURE 2.—The relationship between the linkage groups of the integrated *B. oleracea* map and the cytogenetic map. The linkage groups are represented by vertical bars [percentage of 893 cM (Kosambi) total map length]. The cytogenetic map shows the relative lengths (percentage of haploid complement) and centromeric indices of the chromosomes. Chromosomes 1, 3, 5, 6, and 8 are inverted to follow the orientation of the linkage groups.

(SEBASTIAN *et al.* 2000). To assess data and mapping quality, the segregation data set for each population was ordered by map position and checked for improbable double-recombination events.

BAC-filter hybridization: Gridded filters of the *B. oleracea* BAC (BoB) library of A12DHd (C. D. RYDER and G. J. KING, unpublished data; http://hbz.tamu.edu/bacindex4.html) were screened with probes labeled with ^{32}P following procedures described by VICENTE and KING (2001). BAC clones identified by strong hybridization signals were selected from this fivefold library.

The probes used were pO152; pN2; pR6; pR97, a PCR product of *BoAP1*-b (as described above); a cDNA clone BS29-2 (X16123) kindly supplied by M. Trick (JIC, Norwich), which is homologous to the S-locus gene family; and two other A12DHd genomic PCR products homologous to known gene families.

Assignment of BACs to specific loci: Following initial identification, the BAC clones were subjected to a range of analyses in order to assign them to specific loci. We used a combination of approaches including BAC clone fingerprinting, Southern hybridization, locus-specific PCR, and sequence comparison. The methods used to assign each group of BACs to a specific locus are outlined below and representatives of each group that were subsequently used as FISH probes are shown in Table 1.

Specific BACs had previously been assigned for *BoRGL*-IIIa and *BoRGL*-Va (Vicente and King 2001) and *BoAP1*-c (Smith 1999). BoB001M02 was assigned to *BoRGL*-IVa because it yielded fragments of the expected size when the locus-specific PCR assay described in Vicente and King (2001) was used on candidate BAC templates.

For pO152, pR6, and *BoAP1*-b, positively hybridizing BACs were fingerprinted by DNA extraction and digestion with *Hin*dIII and *Sau*3A followed by fluorescent labeling and electrophoretic separation on an ABI 377 (Applied Biosystems, Foster City, CA). Results were analyzed using Image and FPC software (Sanger Centre) to identify BAC clones with similar fingerprints. BACs identified with pR6 (see http://brassica.bbsrc.ac.uk/update.htm) had similar fingerprints and were assigned to the marker pR6E1. Two of the three pO152 BACs had similar fingerprints. Assignment of the two BAC groups to the markers pO152J1 and pO152E2 was achieved by interpreting the FISH results. The assignment of three BACs, including BoB038G17, to the specific *BoAP1*-b locus was achieved by sequencing a BAC amplicon obtained with the primers developed for the mapping assay.

Sequences homologous to the S-locus gene family were amplified from BACs selected with BS29-2, using primers A and B (Brace *et al.* 1993). The amplicon from one of these BACs, BoB039C15, was used as an RFLP probe on the mapping filters. A single polymorphic band was obtained, which was mapped as marker BoSLGC. The sequences of amplicons from BoB028M02 and BoB030L06 showed closest homology to that of *SLR2* (X57673). A genetic map position for this sequence could not be obtained by using an amplicon as an RFLP probe, due to a lack of polymorphism in the mapping populations.

Using two other gene family-specific probes, five sets of BACs were identified. These BACs were digested with *Eco*RI and used to prepare Southern blots, which were then hybridized with the same gene family probe to reveal locus-specific banding patterns. This enabled identification of locus-specific groups of BACs. For HRI04, the size of the hybridizing fragment in the BAC Southern blot corresponded to the size of the A12DHd allele of the HRI04 RFLP marker. For markers HRI01–HRI03, the PCR product specific for each group of BACs hybridized to a single segregating RFLP. The locus HRI05 was assigned to BoB035N04 because the BAC amplicon sequence and A12DHd genomic DNA sequence were identical.

Plant material and chromosome preparation: Plants of the *B. oleracea* var. *alboglabra* doubled haploid line A12DHd were grown in a glasshouse, with supplementary lighting when necessary, in a 16-hr day length. Preparations of chromosomes were made from anthers, which provide metaphase chromosomes from the mitotic divisions of tapetal cells and meiotic stages from the pollen mother cells. The methods of Armstrong *et al.* (1998) for the fixation of anthers and slide preparation were followed with minor modifications. Anthers were fixed in 3:1 ethanol:glacial acetic acid and washed three times for 2 min in citrate buffer (10 mM, pH 4.5). Digestion in enzyme mixture was reduced to 60–90 min and cytohelicase was sometimes omitted. An anther was broken up in a small drop of water on the slide before the addition of 60% acetic acid and the 45° treatment was reduced to 5–10 sec.

Preparation of FISH probes: The 45S rDNA probe, pTa71 (Gerlach and Bedbrock 1979), EMBL X07841, was directly labeled with a fluorochrome-dUTP (Fluorogreen or Fluorored, Amersham, Buckinghamshire, UK) by nick translation. The 5S rDNA probe, pCT4.2 (Campell *et al.* 1992), was labeled with biotin-16-dUTP by PCR. All other probes were labeled with either biotin-16-dUTP or digoxygenin-11-dUTP by nick translation (Roche, Indianapolis), namely: plasmid clones pW148, pW116, pR6, pO152, pN2, pR97, pNapin36, cosmid clone C14, BACs listed in Table 1, and BAC BoB061G14. The BAC BoB061G14 was a substitute for pBcKB4, which had been used for the partial karyotype (Armstrong *et al.* 1998), as it hybridizes strongly to the pericentromeric heterochromatin of the same six pairs of chromosomes.

C_0t-1 DNA was prepared from A12DHd genomic DNA according to Zwick *et al.* (1997).

Fluorescence *in situ* hybridization: Slides were rinsed in 2× SSC for 10 min, placed in 0.01% pepsin in 10 mM HCl for 1 min, rinsed briefly in distilled water, and fixed in 4% paraformaldehyde for 10 min. They were dehydrated in 70, 85, and 100% ethanol for 2 min each and air dried. Probe mixture (20 μl containing 50% deionized formamide, 2× SSC, 10% dextran sulfate, 50–100 ng labeled probe for each target, and 1 μg C_0t-1 DNA where required) was applied to each slide and sealed under a coverslip with rubber solution. Denaturation at 75° for 4 min was followed by hybridization at 37° overnight in a moist chamber. After removal of the coverslip, slides were washed at 45° three times in 50% formamide 2× SSC, once in 2× SSC, and once in 4× SSC, 0.05% Tween 20 for 5 min each, followed by a further wash in the last solution at room temperature. Detection of digoxygenin-labeled probes was with antidigoxygenin-fluorescein or -rhodamine (Roche) and biotin-labeled probes with Cy3 streptavidin (Cambio, Cambridge, UK). A three-step process with Cy3 streptavidin, biotinylated goat antistreptavidin, and Cy3 streptavidin improved the detection of signals from pNapin36 and RFLP plasmid clones. Slides were counterstained with 4′,6-diamidino-2-phenylindole (1 μg/ml) in Vectashield (Vector, Burlingame, CA) and examined with a Nikon E300 fluorescence microscope. Images were captured and analyzed using an image analysis system (Applied Imaging, Santa Clara, CA), which included a Photometrics Sensys CCD camera and SmartCapture 2 software.

FISH strategy: The existing partial karyotype (Figure 1) provided the basis upon which each linkage group was assigned to a particular chromosome. The 45S probe was used in combination with each of the other probes on mitotic metaphase chromosome preparations. Probes that mapped to the same linkage group were then applied in pairs to confirm that they hybridized to the same chromosome. Chromosome 3 was distinguished from chromosomes 5 and 6, which are smaller but have similar arm ratios, by the fact that the BAC BoB061G14 hybridizes to chromosomes 5 and 6 but not 3. Probes that mapped to different linkage groups but hybridized to chromosomes that were difficult to distinguish using the partial karyotype were applied in pairs to confirm that they hybridized to different chromosomes. If signals from two probes were close together on metaphase chromosomes, the probe order was confirmed using meiotic pachytene spreads. The orientation of linkage groups with respect to the chromosomes was achieved through use of two or more FISH landmarks and chromosome morphology.

TABLE 1

Assignment of linkage groups to chromosomes with FISH probes

Linkage group	Chromosome	Locus	Marker assay	FISH probe	Vector	C_0t-1 DNA used
O1	8	pNapin36	RFLP	pNapin36	Plasmid	No
O1	8	BoRGL-Va	CAPS	BoB013D19	BAC	Yes
O2	5	BoAP1-b	RFLP	BoB038G17	BAC	Yes
O2	5	HRI01	RFLP	BoB014O06	BAC	No
O2	5	HRI04	RFLP	BoB006C04	BAC	Yes
O3	1	HRI05	SNP	BoB035N04	BAC	Yes
O3	1	pW148E1	RFLP	pW148	Plasmid	No
O3	1	pR6E1	RFLP	BoB056C17	BAC	Yes
O4	2	Bo45SIGS	RFLP	pTa71	Plasmid	No
O4	2	BoRGL-IIIa	CAPS	BoB011O13	BAC	Yes
O5	6	pO152J1	RFLP	BoB013F17	BAC	Yes
				BoB014L04	BAC	Yes
O6	9	BoAP1-c	CAPS	BoB028L01	BAC	Yes
O6	9	[a]		BoB028I05	BAC	Yes
O7	4	HRI02	RFLP	BoB021L05	BAC	No
				BoB034L12	BAC	No
O7	4	BoSLGC	RFLP	BoB039C15	BAC	No
O8	7	BoRGL-IVa	CAPS	BoB001M02	BAC	Yes
O8	7	pO152E2	RFLP	BoB023L12	BAC	Yes
O9	3	HRI03	RFLP	BoB012H11	BAC	Yes
				BoB040I15	BAC	Yes
O9	3	pC14E1	RFLP	C14	Cosmid	No

[a] Positioned by physical mapping, hence no genetic map locus.

RESULTS

Genomic clones of different lengths provide single-copy FISH signals: We obtained clear FISH signals with probes of different lengths from a range of sources (Table 1). These included a genomic RFLP clone of 1880 bp, a cDNA plasmid clone with a 3.3-kb insert, a cosmid clone with a 30-kb insert, and BAC clones with inserts from 45 to 120 kb.

Many BAC clones required the inclusion of C_0t-1 DNA in the probe mixture. Because C_0t-1 DNA blocked the hybridization of the repetitive DNA in the BAC clones, signals from nonspecific hybridization sites were reduced and the signal from the specific hybridization site of the probe could be distinguished more easily. Of the 17 BACs placed on both the cytogenetic and genetic maps, only four gave clear signals without C_0t-1 DNA, and of these, two represented the same marker (Table 1). No specific hybridization sites could be determined confidently for two BACs, even with C_0t-1 DNA. One of these, BoB061G14, hybridized strongly to the pericentromeric heterochromatin of six pairs of chromosomes and, although the signals obtained with this BAC were significantly reduced with increasing amounts of C_0t-1 DNA, the specific hybridization site could not be identified. The signals produced by the second BAC, BoB060E03, were scattered across the chromosomes, but all these signals were reduced when C_0t-1 DNA was used, suggesting that a large proportion of this BAC consisted of repetitive DNA.

Of the six genomic plasmid clones with small inserts used as FISH probes, namely pW116, pW148, pO152, pN2, pR6, and pR97, only the largest, pW148, with an insert size of 1880 bp, gave signals that could be consistently identified. When labeled with biotin-16-dUTP this clone could not be detected directly using Cy3 but, using a three-step Cy3 streptavidin detection procedure, double signals on one pair of chromosomes were detected. The double signal is produced as the probe hybridizes to both chromatids. Background signals, which are frequently observed in FISH preparations, are more noticeable when detecting weak signals from small probes as the signal-to-noise ratio is low. In Figure 3C, signals other than those marked as specific to the probe pW148 are visible, but consideration of many metaphase spreads confirmed them to be background signals as they were single and did not appear consistently in the same positions. Although the A12DHdxGDDH33 map has two RFLP markers associated with the clone pW148, the detection of only one pair of signals was not unexpected since the RFLP autoradiograph showed that A12DHd had a null allele for one of the markers. This indicated that pW148 would hybridize to one locus only when used as a FISH probe on A12DHd chromosomes.

The hybridization of the biotin-16-dUTP-labeled cDNA

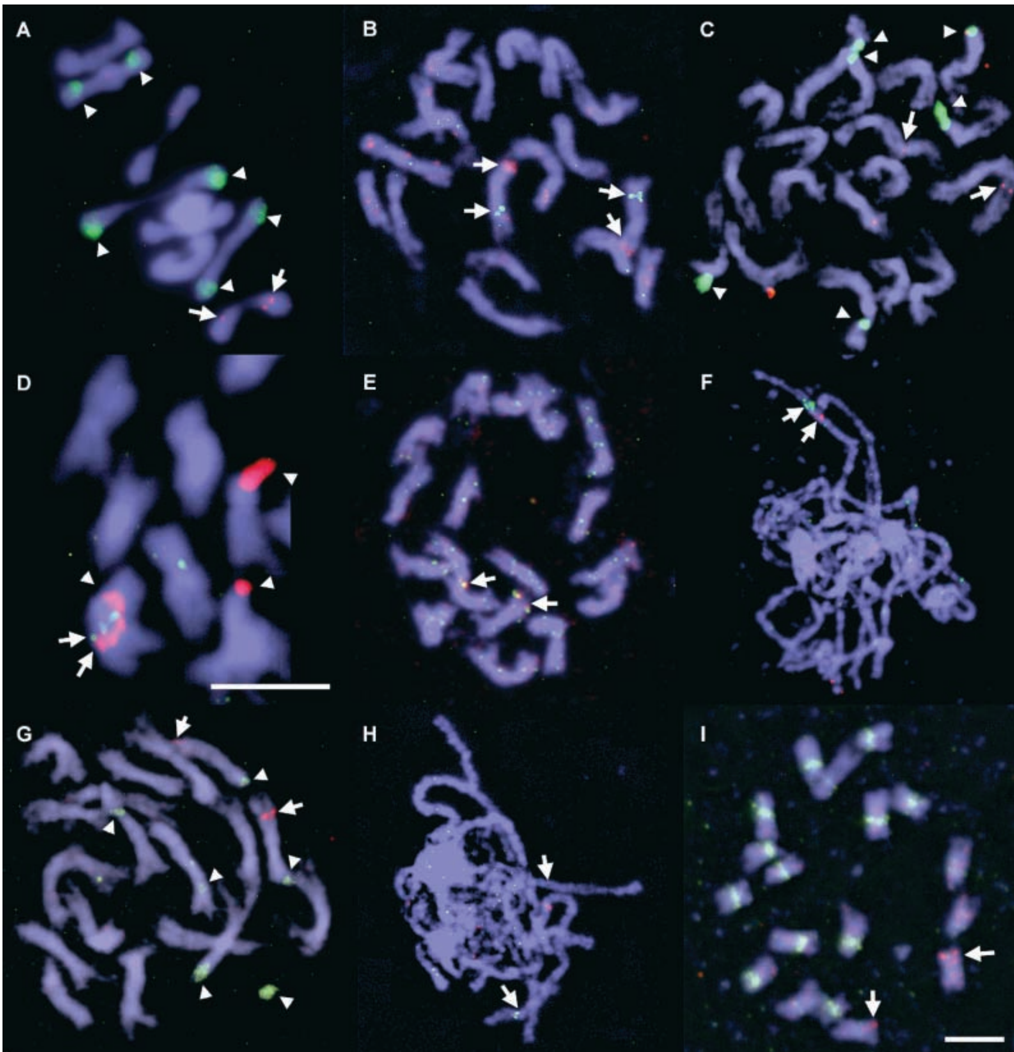

Figure 3.—Examples of FISH with 45S rDNA (arrowheads) and other probes (arrows) on *B. oleracea*. (A) LGO1 meiotic diakinesis, pNapin36 (red). (B) LGO2 mitotic metaphase, BoB014-O06 (red) and BoB038G17 (green). (C) LGO3 mitotic metaphase, pW148 (red). (D) LGO4 partial mitotic metaphase, 5S rDNA (red) and BoB011O13 (green). (E) LGO5 mitotic metaphase, BoB013F17 (red) and BoB014L04 (green), over-lapping. (F) LGO6 meiotic pachytene, BoB028L01 (red) and BoB028I05 (green). (G) LGO7 mitotic metaphase, BoB021L05 (red). (H) LGO8 meiotic pachytene, BoB001-M02 (red) and BoB023L12 (green). (I) LGO9 mitotic metaphase, cosmid C14 (red) and BoB061G14 (green). (A–C, E–I) Bar, 10 μm. (D) Bar, 10 μm.

plasmid clone pNapin36 with a 3.3-kb insert could be detected with Cy3, but the frequency of detection and strength of signal was improved by the amplification of the signal in the three-step procedure.

In a preliminary screen of six cosmids, C14 was the only one for which a specific hybridization site could be detected without the use of C_0t-1 DNA. Since the others were not mapped, only C14 was used in this investigation and it gave a strong signal on the short arm of each chromatid of a pair of metacentric chromosomes with either antidigoxygenin-fluorescein or Cy3 streptavidin.

Strategy to assign genetic loci to chromosomal positions: We began by investigating the possibility of using plasmids and cosmids with genetically mapped inserts as FISH probes to link the genetic markers with their chromosomal position. Only one of six RFLP plasmids with inserts between 1500 and 1880 bp was used successfully as a FISH probe and this required a more time-consuming detection system than did longer probes. One cDNA clone with a 3.3-kb insert gave a clear FISH signal and we were able to map this gene, which encoded a napin seed storage protein to linkage group O1. One mapped cosmid clone was also successfully used as a FISH probe. However, due to the lack of availability of such clones having longer inserts than those of the plasmids from the pW, pO, pN, and pR libraries as well as map positions on the integrated map, we decided to concentrate on the use of genomic BAC clones. The initial FISH results with BAC clones from the "BoB" library constructed from A12DHd indicated that many would produce a clear, strong site-specific signal, provided that C_0t-1 DNA was included to block repetitive sequences and would thus be ideal chromosome markers.

However, to use BACs effectively it was necessary to assign them unequivocally to specific loci. This required a variety of approaches depending on the locus, as detailed in materials and methods. Three BACs had already been assigned to the specific genetic loci of *BoRGL*-IIIa, *BoRGL*-Va, and *BoAP1*-c and a candidate BAC was confirmed for *BoRGL*-IVa. Probing the BAC library with four of the RFLP plasmids, pO152, pN2, pR6, and pR97, provided BACs for markers pO152J1, pO152E2, and pR6E1 but pN2 and pR97 failed to hybridize to any BACs. We also mapped the *BoAP1*-b locus,

which is the third member of this MADS-box gene family to be mapped in the *B. oleracea* genome, and assigned BACs to this locus. Three gene family probes, including one for the S-locus gene family, identified BACs that we assigned to six loci. These loci were added to the integrated map and included *BoSLGC*, which is a putative additional member of the S-locus gene family.

The 14 loci for which we had confirmed BAC clones, together with pW148E1, pC14E1, pNapin36, and a 45S locus, which we were able to map, were distributed among the linkage groups. The BAC BoB028I05 was suggested to be proximate to BoB028L01 on linkage group O6, on the basis of evidence from ongoing physical mapping (our unpublished data). This was confirmed by FISH and provided a locus on O6 assigned to the karyotype.

Assignment of linkage groups to chromosomes: The comprehensive assignment of the linkage groups to the chromosomes (Figure 2) was achieved as follows.

Linkage group O1: The plasmid clone pNapin36 was mapped as an RFLP to linkage group O1 and, when used as a FISH probe, it hybridized to the long arm of chromosome 8, one of the smallest chromosomes in the *B. oleracea* karyotype (Figure 3A). BoB013D19, a BAC containing *BoRGL*-Va that had previously been located on O1 (Vicente and King 2001), hybridized to the short arm of chromosome 8.

Linkage group O2: Three BACs hybridized to chromosome 5. Each BAC was assigned to linkage group O2 using different methods (see materials and methods). HRI01 and HRI04 map adjacent to each other and their associated BACs both locate on the short arm of chromosome 5. A confirmed single locus BAC for *BoAP1*-b (BoB038G17) gave a FISH signal on the long arm of chromosome 5 (Figure 3B).

Linkage group O3: The clone pW148 mapped to linkage groups O3 and O2, although there was a null allele in A12DHd for the RFLP marker on linkage group O2. FISH signals were detected at a single locus on the long arm of chromosome 1, indicating that chromosome 1 corresponds to linkage group O3 (Figure 3C). BAC BoB056C17, identified with clone pR6, also hybridized to a site on the long arm of chromosome 1, closer to the pericentromeric heterochromatin than pW148. The BAC BoB035N04 associated with HRI05 hybridized close to BoB056C17. On meiotic pachytene preparations, BoB035N04 was confirmed to hybridize farther from the pericentromeric heterochromatin than BoB056C17.

Linkage group O4: The locus *BoRGL*-IIIa had previously been mapped to linkage group O4 and the BAC BoB011O13 was shown to contain this locus-specific sequence (Vicente and King 2001). This BAC hybridized to chromosome 2 between the 45S rDNA and the 5S rDNA sites (Figure 3D).

When the Bo45SIGS PCR product was used as an RFLP probe, several monomorphic bands and a single polymorphic band were detected. The polymorphic band was present in A12DHd but absent in GDDH33 and genetic mapping placed this marker on linkage group O4. The plasmid pTa71, used as a FISH probe for 45S rDNA, hybridizes to chromosomes 4 and 7 in GDDH33 and A12DHd but it also hybridizes to a site on chromosome 2 in A12DHd. It was thought that the marker on linkage group O4 would correspond to this site on chromosome 2 and the association of linkage group O4 with chromosome 2 by BAC BoB011O13 supports this view.

Linkage groups O5 and O8: The locus *BoRGL*-IVa had previously been mapped to linkage group O8 (Vicente and King 2001). The BAC BoB001M02 was subsequently shown to contain the corresponding locus-specific sequence and it hybridized to chromosome 7. pO152 has two loci, which are on linkage groups O5 and O8. Fingerprinting of the BACs identified by hybridization with pO152 revealed that BAC clones BoB013F17 and BoB014L04 were contiguous with each other but not with BoB023L12. When used as a FISH probe, BoB023L12 produced a strong signal on chromosome 7 and was therefore considered to be the BAC associated with pO152E2 on linkage group O8. BoB023L12 and BoB001M02 are shown on the same chromosome of a meiotic pachytene preparation in Figure 3H.

The remaining BACs identified with pO152, namely, BoB013F17 and BoB014L04, co-located on a medium-sized metacentric chromosome (Figure 3E). By a process of elimination, incorporating data from other chromosomes and linkage groups, this chromosome was identified as chromosome 6 and hence could be assigned to linkage group O5.

Linkage group O6: BoAP1-c was a CAPS marker that had previously been mapped to linkage group O6, while an identical sequence was detected within the BAC BoB028L01 (Smith 1999). This BAC hybridized to the long arm of chromosome 9. BoB028I05 is an additional BAC that has been identified during development of a physical map contig in the region (our unpublished results) by exploiting the collinearity between sections of linkage group O6 and *A. thaliana* chromosome 1 (Ryder *et al.* 2001). This BAC was placed on linkage group O6 distal to BoB028L01 (*BoAP1*-c; G. C. Barker and G. J. King, unpublished data). FISH results confirmed that the two BACs were on the same chromosome, with BoB028I05 in the more distal position (Figure 3F). This was the first situation in which we were able to demonstrate the coincidence of two BACs on the same chromosome on the basis of evidence from physical mapping rather than genetic linkage.

Linkage group O7: HRI02 was an RFLP marker that mapped to linkage group O7. There was no evidence of additional loci from the RFLP autoradiograph. Fingerprinting showed that BACs BoB021L05 and BoB034L12 overlap and their identity in relation to HRI02 was confirmed by sequence comparison. When used as FISH probes, both BACs located on the long arm of chromo-

some 4, a chromosome with a distal 45S rDNA site. BoB021L05 is shown hybridizing to chromosome 4 in Figure 3G.

BoSLGC was a unique RFLP fragment derived from BoB039C15, which mapped to linkage group O7. BoB039C15 hybridized to chromosome 4 on the same arm and proximal to the centromere in relation to BoB021L05.

Linkage group O9: The cosmid clone C14 mapped to linkage group O9. The same probe hybridized to the shorter arm of chromosome 3 (Figure 3I). HRI03 was an RFLP marker that mapped to linkage group O9. Two BACs, BoB012H11 and BoB040I15, were associated with HRI03 and their identity was confirmed by fingerprinting and sequence comparison. Both BACs hybridized to the same position on the longer arm of chromosome 3.

Orientation of linkage groups with chromosomes: The location of more than one probe on each of eight chromosomes allows comparison of the orientation of chromosomes and linkage groups. The chromosomes are displayed in Figure 1 according to cytogenetic convention with the short arm at the top of the vertical chromosome. In Figure 2, the linkage groups are displayed as they were in the A12DHdxGDDH33, NxG, and integrated maps (Bohuon *et al.* 1996; Sebastian *et al.* 2000) while the chromosomes are inverted, when applicable, to match this orientation. Chromosomes 2, 4, 7, and 9 are in the same orientation as their respective linkage groups (O4, O7, O8, and O6), whereas chromosomes 1, 3, 5, and 8 are in the opposite orientation to linkage groups O3, O9, O2, and O1, respectively. Chromosome 6 cannot be oriented with respect to linkage group O5 until another probe can be located on the chromosome. However, chromosome 6 has been inverted in Figure 2 because the location of the current FISH probe and map position of the marker suggest that the chromosome and linkage group are in opposite orientation.

DISCUSSION

We have assigned all nine linkage groups of a *B. oleracea* genetic map to each of the nine chromosomes of the karyotype derived from mitotic metaphase spreads from tapetal cells of the *B. oleracea* var. *alboglabra* line A12DHd using FISH. Great care was taken to ensure that the individual BACs used as FISH probes in this study corresponded to the same locus as the genetically mapped sequence. This step is particularly important for this species because more than one copy of many genes and regions of the genome are present (Lagercrantz and Lydiate 1996; Lan *et al.* 2000).

A low rate of success was achieved when using plasmid clones with small inserts directly as FISH probes. This may be due to a variable accessibility of the DNA on the chromosomes. It is also possible that the successful probe, pW148, may be detecting a locus duplicated in tandem, which would provide a longer hybridization site and therefore a stronger signal. The size of these inserts was close to the minimum length of 1.3 kb used successfully as a FISH probe for a single-copy sequence in plants (Ohmido *et al.* 1998). We did not attempt to improve the detection of small probes because the detection system would be more time consuming than that used for BACs and we decided that BACs with strong site-specific signals would be preferable as chromosome markers.

Regions of the Brassica genomes are collinear with regions in *A. thaliana* (Cavell *et al.* 1998; Ryder *et al.* 2001), and many *A. thaliana* loci are present as three or more copies in the diploid Brassica species (Kowalski *et al.* 1994; Lagercrantz 1998). It is therefore not surprising to find that BAC probes (made up of fragments produced by nick translation) sometimes hybridize to more than one site on the Brassica chromosome complement. However, when more than one signal was present, we consistently observed that the chromosome-marker BACs gave one noticeably stronger signal. The weaker signals are presumed to result from hybridization of the probe fragments to any other regions having good homology, such as loci paralogous to the sequence for which a BAC has been selected, loci paralogous to other genes on the BAC, and dispersed repeats if these are not fully blocked. In such cases, it is possible that the paralogous segments are shorter than the BAC insert and/or that homology over the whole segment is not sufficient for hybridization to occur to the same extent as at the main site. This agrees with a study at the microgenome level in which regions of the *B. oleracea* genome homeologous to a 222-kb region of *A. thaliana* were identified (O'Neill and Bancroft 2000). Triplication and collinearity were confirmed but there was a high frequency of gene content variation between the paralogous segments of *B. oleracea* as well as between them and the *A. thaliana* segment. Differences between paralogous segments in *B. rapa* have also been indicated in a study using FISH (Jackson *et al.* 2000). A 431-kb Arabidopsis BAC contig used as a single FISH probe on *B. rapa* metaphase chromosomes generated signals on four to six chromosomes. The authors suggested that some of the multiplied loci may have undergone significant rearrangements resulting in the inconsistent number of foci in different cells. When the BACs were used in fiber-FISH, the standard deviations of measurements of signals from two of the four BACs were much larger in *B. rapa* compared to *A. thaliana*, indicating that the measured signals represented more than one locus in the *B. rapa* genome and these loci had different physical sizes.

The numbering of the chromosomes in the cytogenetic karyotype was based on rank lengths, excluding the lengths of the satellites (Armstrong *et al.* 1998), but this numbering does not correspond to other karyo-

types of *B. oleracea* because different conventions were followed (Cheng *et al.* 1995; Fukui *et al.* 1998). The assignment of the cytogenetic map to the genetic map now provides a framework to align karyotypes previously published by different research groups. The longest chromosome of the *B. alboglabra* karyotype of Cheng *et al.* (1995) is their chromosome 4 and it carries the gene controlling flower color. The longest chromosome (A) of the *oleracea*-type chromosomes in the *B. napus* karyotype of Kamisugi *et al.* (1998) hybridized to a probe for the gene *SLR1*. Our longest chromosome, chromosome 1, corresponds to linkage group O3, to which the gene for flower color and the gene *SLR1* have been mapped (Ramsay *et al.* 1996). The probe for the *SLG* gene hybridized to the shortest of the *oleracea*-type chromosomes (Kamisugi *et al.* 1998). This gene has been mapped to linkage group O6 (Ramsay *et al.* 1996), which is our shortest chromosome, chromosome 9. There is also one instance in which information from the cytogenetic map provides additional evidence for the alignment of two linkage groups from different genetic maps. Hu *et al.* (1998) aligned linkage groups from their base map with those of three other, independent, *B. oleracea* RFLP genetic maps. Using the data they presented and comparing it to our current integrated map it is possible to equate R8 (Ramsay *et al.* 1996), which is our linkage group O8, to their C6. They present a rDNA locus at one end of C6, and using FISH we have found that linkage group O8, chromosome 7, has a distal 45S rDNA site.

Genetic maps are scaled in proportion to the relative frequency of recombination. Comparing our cytogenetic and genetic maps, it can be seen that the longest linkage group is also the longest chromosome and the shortest linkage group is the shortest chromosome. However, it appears that linkage groups O7 and O8 are shorter than might be expected from the length of the corresponding chromosomes. They also both have clusters of markers near the top (http://www.biology.bham.ac.uk/brassica_map/). It is possible that recombination is reduced near the large 45S loci on these chromosomes or that the linkage groups do not extend to the end of the chromosomes due to a lack of polymorphic markers. At present, only general observations can be made regarding the relationship between the positions of probes on the genetic map compared to the cytogenetic map, because few probes have been placed on the cytogenetic map and their positions are not based on systematic measurement. If linkage group O2/chromosome 5 is considered, the physical distance on a metaphase chromosome between BoB038G17 (*BoAP1*-b) and BoB014O06 (HRI01) is much greater than the distance from the end of the chromosome to BoB038G17 (Figures 2 and 3B), but on the genetic map BoAP1-b is approximately equidistant between the end and HRI01. Since the centromere occurs between these probes, it is possible that this difference is a result of recombination being reduced in proximal as compared to distal regions of the chromosome, as is the situation with other species such as wheat and barley (Künzel *et al.* 2000; Sandhu *et al.* 2001). In one case, two probes (BoB035N04 and BoB056C17) are in a different order on the cytogenetic map compared to the order of the corresponding markers (HRI05 and pR6E1) on linkage group O3 of the integrated map. The two markers were mapped in different populations and upon integration of the maps they were placed only 1.6 cM apart in a region where joint markers showed no rearrangements of order. It is likely that the cytogenetic order is correct for A12DHd whereas the genetic order may be due to an artifact of the mapping process.

We are currently carrying out a more detailed comparison of genetic and physical distances over a specific region of one chromosome, using the less condensed chromosomes of the meiotic pachytene stage. An estimate of the physical distance between probes is valuable when positional cloning is being considered. It should also be possible to estimate the physical length of a region of DNA from a donor parent in a substitution line by using FISH probes associated with markers at each end of the substitution. A set of substitution lines is available for A12DHd with GDDH33 as the donor parent and is being used for QTL analysis (Ramsay *et al.* 1996; Rae *et al.* 1999).

We have already used the chromosome-specific markers and the FISH technique to assign BACs to particular linkage groups. For example, BoB028M02, containing the sequence for the S-locus related gene *SLR2*, was shown to hybridize to chromosome 1 when used in combination with a specific marker for this chromosome and therefore has been assigned to linkage group O3. Because the marker assay for *SLR2* was monomorphic in our mapping populations we have no genetic map position for this gene. Once additional chromosomal markers with known genetic positions are identified, it will be possible to place less characterized markers not only in a linkage group but also in a particular region of that linkage group. During our investigation four BACs with A12DHd inserts were applied to pachytene preparations from a cauliflower and a broccoli cultivar and each gave a strong site-specific signal. This suggests that BACs with inserts from other *B. oleracea* cultivars may similarly produce signals on A12DHd preparations and the position of the DNA homologous to these inserts can be determined within the context of the A12DHd genome.

The BAC library is currently being used in a program to generate a physical map of *B. oleracea* (http://brassica.bbsrc.ac.uk), drawing on the complete genomic sequence available for *A. thaliana* and its close relationship to *B. oleracea*. The technique of FISH together with the cytogenetic map should prove a useful tool in situations where assignment of BACs to an overlapping set of contiguous clones (contig), or contigs to linkage groups, is difficult.

We are grateful to T. Osborn, University of Wisconsin, and to Advanta for permission to use Brassica hybridization probes. We also acknowledge R. Naylor (HRI) for the BAC library and RFLP hybridizations, D. Patel (HRI) for generating fingerprint data, and S. Price (Birmingham University) for valuable technical assistance. This work was funded by the UK Biotechnology and Biological Sciences Research Council. E. P. Kop was supported by Vilmorin-Clause et Cie and J. G. Vicente was supported by a grant from PRAXIS XXI (BD/3780/94).

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Communicating editor: C. Haley