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Wide-Cross Whole-Genome Radiation Hybrid Mapping of Cotton (*Gossypium hirsutum* L.)

Wenxiang Gao,* Z. Jeffrey Chen,* John Z. Yu,† Dwaine Raska,* Russell J. Kohel,† James E. Womack‡ and David M. Stelly*,[1]

**Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas 77843-2474, †USDA-ARS, Crop Germplasm Research Unit, College Station, Texas 77845 and ‡Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas 77843*

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ABSTRACT

We report the development and characterization of a "wide-cross whole-genome radiation hybrid" (WWRH) panel from cotton (*Gossypium hirsutum* L.). Chromosomes were segmented by γ-irradiation of *G. hirsutum* ($n = 26$) pollen, and segmented chromosomes were rescued after *in vivo* fertilization of *G. barbadense* egg cells ($n = 26$). A 5-krad γ-ray WWRH mapping panel ($N = 93$) was constructed and genotyped at 102 SSR loci. SSR marker retention frequencies were higher than those for animal systems and marker retention patterns were informative. Using the program RHMAP, 52 of 102 SSR markers were mapped into 16 syntenic groups. Linkage group 9 (LG 9) SSR markers BNL0625 and BNL2805 had been colocalized by linkage analysis, but their order was resolved by differential retention among WWRH plants. Two linkage groups, LG 13 and LG 9, were combined into one syntenic group, and the chromosome 1 linkage group marker BNL4053 was reassigned to chromosome 9. Analyses of cytogenetic stocks supported synteny of LG 9 and LG 13 and localized them to the short arm of chromosome 17. They also supported reassignment of marker BNL4053 to the long arm of chromosome 9. A WWRH map of the syntenic group composed of linkage groups 9 and 13 was constructed by maximum-likelihood analysis under the general retention model. The results demonstrate not only the feasibility of WWRH panel construction and mapping, but also complementarity to traditional linkage mapping and cytogenetic methods.

GENOME maps are used extensively for comparative, phylogenetic, and evolutionary genomics; map-based gene cloning; trait dissection; and marker-assisted molecular breeding. Comprehensive linkage maps have been developed for most major crops such as rice (*Oryza sativa* L., $2n = 24$; Goff *et al.* 2002; Yu *et al.* 2002), corn (*Zea mays* L., $2n = 20$; http://www.maizemap.org/), and bread wheat (*Triticum aestivum* L., $2n = 42$; http://wheat.pw.usda.gov/ggpages/maps.shtml#wheat). For cotton, significant progress has been made in public linkage map development (http://demeter.bio.bnl.gov/acecot.html; Reinisch *et al.* 1994; Shappley *et al.* 1998; Yu *et al.* 1998; J. Z. Yu and R. J. Kohel, unpublished data; Ulloa and Meredith 2000; Zhang *et al.* 2002; Lacape *et al.* 2003; Mei *et al.* 2004), but major improvements are needed. Physical coverage of the cotton (*Gossypium hirsutum* L.) genome by these linkage maps remains unknown. The number of linkage groups exceeds the gametic chromosome number ($n = 26$), and numerous linkage groups are yet to be associated with specific chromosomes. Moreover, common identities have yet to be established among many linkage groups in the laboratory-specific maps, and a common nomenclature is yet to be established. These and other impediments to cotton genomics might be ameliorated by radiation hybrid mapping, which has greatly catalyzed development of animal genomics over the past decade.

Goss and Harris (1975) first used radiation-induced chromosome rearrangements to map genes on human chromosome X. After lethal X-ray irradiation of human cell lines, fragmented human X chromosomes were rescued by rodent cells by means of cell fusions. In contrast to natural recombination, radiation-induced recombination frequencies and map resolution can be modulated by modifying radiation doses. However, this technique for physical mapping was not widely employed until Cox *et al.* (1990) used radiation hybrids to construct a high-resolution map of human chromosome 21. This new mapping technology, radiation hybrid (RH) mapping, not only increased the recombination events, but also offered the advantages of very high rates of polymorphism between donor and recipient cell lines. Markers that were otherwise monomorphic and essentially unusable for mapping were thereby rendered polymorphic and usable (Cox *et al.* 1990).

A major limitation of the RH mapping method of Cox *et al.* (1990), however, is that it maps only one

[1]*Corresponding author:* Department of Soil and Crop Sciences, Texas A&M University, 370 Olsen Blvd., College Station, TX 77843-2474. E-mail: stelly@tamu.edu

chromosome at a time. It is difficult to generate a high-resolution map of organisms with many chromosomes (Walter *et al.* 1994). To overcome this limitation, Walter *et al.* (1994) reported an improved RH mapping method, termed whole-genome radiation hybrid (WGRH) mapping. In WGRH mapping, whole-genome radiation hybrids are generated when the donor material is obtained from a diploid cell line of the donor species, rather than from a somatic cell hybrid single-chromosome addition line (Cox *et al.* 1990; Walter *et al.* 1994). As a result, the WGRH mapping method allows all chromosomes to be mapped using a single radiation hybrid panel (Walter *et al.* 1994). The WGRH mapping approach has been rapidly assimilated into genome mapping efforts for humans and certain animal species (McCarthy 1996; Womack *et al.* 1997).

In plants, radiation treatments have been used mainly for inducing mutation (Driscoll and Jensen 1963; Kohel 1973; Islam *et al.* 1981; Riley and Law 1984; reviewed in Ahloowalia and Maluszynski 2001). Radiation treatments have been used to mutate genes and to generate chromosome translocations and aneuploids in cotton (Kohel 1973; Endrizzi *et al.* 1985; D. M. Stelly, unpublished data). RH mapping of an oat-maize addition line was recently used to map maize chromosome 9 (Riera-Lizarazu *et al.* 2000), *i.e.*, where the scope of mapping for each radiation hybrid panel is relegated to a single chromosome, similar to the RH method of Cox *et al.* (1990). Recently, Wardrop *et al.* (2002) reported *in vitro* WGRH cell line hybrids developed by incorporation of barley (*Hordeum vulgare*) genome fragments into tobacco (*Nicotiana tabacum*) protoplasts. The difficulty of finding appropriate recipient cell lines to rescue irradiation-fragmented plant chromosomes is a major obstacle in the application of RH or WGRH in mapping plant species. To circumvent this limitation, we instead used a different but related species to rescue irradiated chromosomal segments. Rather than introduce fragmented chromosomes *in vitro* and deal with extensively mosaic cell populations, as in traditional RH or WGRH mapping methods, we used the genome of one species to rescue irradiated chromosomal segments of another species within the same genus through wide-crossing. We termed this modified WGRH mapping approach as wide-cross whole-genome radiation hybrid (WWRH) mapping. The cultivated cotton species *G. hirsutum* and *G. barbadense* were used to test the feasibility of the WWRH mapping method, because they are relatively tolerant of hypoaneuploidy. In this article, we present results on WWRH in cotton, which indicate it to be a promising mapping approach that will help improve the overall cotton genome map.

MATERIALS AND METHODS

Plant materials: One accession from each of two tetraploid cotton species was chosen as parents to generate a WWRH panel: the female parent 3-79 is a doubled haploid line of *G. barbadense* L. ($2n = 52$) with two $(AD)_2$ genomes, and pollen parent TM-1 is a highly inbred line of *G. hirsutum* L. ($2n = 52$) with two $(AD)_1$ genomes. They were selected as parents because they have been used extensively for linkage mapping (http://demeter.bio.bnl.gov/acecot.html; Yu *et al.* 1998; J. Z. Yu and R. J. Kohel, unpublished data), tolerate aneuploidy, and are homozygous or largely homozygous. Relatively high molecular marker polymorphism exists between them, and both are cultivated forms of cotton.

Radiation treatment: TM-1 flowers at anthesis were irradiated with γ-rays at the Texas A&M University Nuclear Science Center (NSC) or the College of Veterinary Medicine (CVM) and then used to pollinate 3-79 flowers emasculated the day before. Four radiation dosages, 1.5 and 5 krad at NSC and 15 and 30 krad at CVM, were used to irradiate TM-1 flowers. For 1.5- and 5-krad treatments, ~150 cross-pollinations were made, and for 15- and 30-krad treatments, ~80 cross-pollinations were made. The number of F_1 seeds, F_1 seed germination frequency, and F_1 plant chromosome deletion types and deletion frequencies were surveyed as described below. The data were used to select which dosage might be the "best" for constructing a WWRH mapping panel, where desirable features include relatively high frequencies of paternal segmental deletions and sufficient recovery of viable F_1 seeds and F_1 plants.

Radiation hybrid genotyping and 5-krad WWRH mapping panel: Thirty-three simple sequence repeat (SSR) markers from four chromosomes and one linkage group were used to genotype and characterize the pilot WWRH panels. Up to 22 WWRH plants were included in the radiation dosage pilot-screening population for each of the dosages. A WWRH mapping panel composed of 93 RH plants was constructed for the selected radiation dosage (5 krad) and was genotyped at more SSR marker loci. Experimental controls included the two parents, 3-79 and TM-1, as well as a nonirradiated 3-79 × TM-1 F_1 hybrid.

DNA samples were extracted from young leaf tissues of ~2-month-old plants. SSR primers were purchased from Research Genetics (Birmingham, AL). Each PCR reaction included 100 ng genomic DNA, 0.2 mm dNTPs, 3.0 mm $MgCl_2$, 0.04 μm forward primer, 0.04 μm reverse primer, and 0.5 unit of Taq polymerase in 15 μl total reaction volume. PCR reaction was performed as follows: 95° for 2 min; 40 cycles of 94° for 45 sec, 55° for 45 sec, 72° for 1 min; 72° for 7 min; and 4° for holding. PCR products were resolved in 4% agarose gels. WWRHs missing TM-1 PCR amplification products were identified as deletion lines; that is, the TM-1 chromosomal segments containing corresponding SSRs were deleted from those WWRHs (Figure 1). All 5-krad WWRHs (101 in total) were genotyped twice for given SSR markers from LG 9 (J. Z. Yu and R. J. Kohel, unpublished data). In the second round of genotyping, all conditions were the same as that mentioned above, except that the total PCR reaction volume was increased to 30 μl. All other SSR markers were genotyped only once on the constructed 5-krad WWRH mapping panel, which included 93 randomly chosen WWRHs, each parent, and a non-irradiated hybrid (control).

Statistical analysis: The presence/absence (+/−) of each marker was scored for each WWRH, and a question mark was assigned to a radiation hybrid when its marker pattern was uncertain. The retention frequency for a given marker was calculated simply as the ratio of number of WWRH plants carrying the marker band to the total number of WWRH plants unambiguously screened in that panel. The radiation dosage deemed to offer the best combination of high plant viability and high marker deletion was chosen for more detailed characterization of a given linkage group. For that purpose, we used SSR markers of LG 9. Chi-square tests were

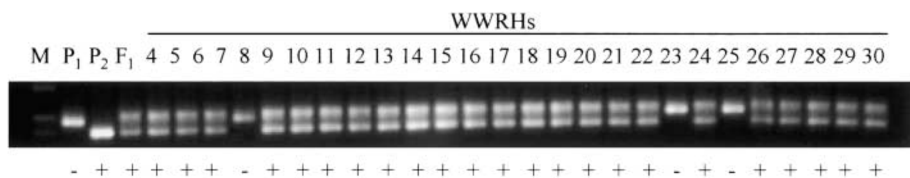

Figure 1.—Detection of WWRH deletion lines. PCR products of SSR marker BNL1066 were resolved in a 4% agarose gel. Lane 1, a molecular ladder (M); lane 2, *G. barbadense* line 3-79 (P_1); lane 3, *G. hirsutum* line TM-1 (P_2); lane 4, normal F_1 hybrid from 3-79 × TM-1; lanes 5–30, WWRHs. All WWRHs (lanes 5–30) had bands from female parent 3-79. Plus and minus indicate the present and absent band patterns of PCR products from the pollen parent, respectively. WWRHs in lanes 8, 23, and 25 indicated missing bands of PCR products from TM-1 and were identified as WWRH deletion lines.

carried out to determine if SSR marker retention frequency was independent of its location in LG 9. LG 9 was arbitrarily separated into eight segments, each represented by one SSR marker on it. Marker retention frequency and pattern were used to represent the corresponding chromosomal segment retention frequency and pattern. Markers that had been colocalized in the linkage map were logically ordered by radiation hybrid analysis according to marker retention patterns in the WWRH panel and the minimum obligate breaks approach. The multiple-point radiation hybrid mapping program RHMAP version 2.01 (Boehnke *et al.* 1995) was used to generate a WWRH map.

RHMAP analysis: Genotypic data from 102 SSR markers were used to run the RHMAP program to test the feasibility of generating a WWRH map. Because only 60 markers could be used in running the RHMAP program at one time, several rounds of two-point RHMAP analyses were carried out using genotypic data from 60 of 102 SSR markers at one time to identify syntenic groups. The minimum LOD score of four was set for a significant syntenic group. On the basis of two-point analysis, we chose the syntenic group containing the most marker loci for further analysis. This group of marker loci was subjected to maximum-likelihood analysis using the RHMAP program to construct a WWRH map.

Integrative mapping and cytogenetic aneuploid analysis: The WWRH syntenic groups generated from RHMAP analyses were compared and contrasted with those from conventionally developed molecular marker linkage maps (http://demeter.bio.bnl.gov/acecot.html; Yu *et al.* 1998; J. Z. Yu and R. J. Kohel, unpublished data). In current cotton linkage maps, not all linkage groups have been associated with chromosomes and the linkage group number exceeds that of gametic chromosome number. When we associated an SSR marker from a linkage group with SSR marker(s) from a syntenic group in our WWRH map, the linkage group was assigned to that syntenic group. When two or more SSR markers from different linkage groups were found by WWRH mapping to be syntenic, the corresponding linkage groups were combined. To test the assignments of different linkage groups to a common syntenic group, markers from the respective linkage groups were tested against appropriate hypoaneuploid interspecific hybrid stock(s), if available. The development and application of cotton hypoaneuploid stocks for chromosome assignments have been described previously (Reinisch *et al.* 1994; Mei *et al.* 2004).

RESULTS

Dosage effect analysis: The number and quality of seeds resulting from cross-pollination with γ-irradiated pollen differed markedly across the radiation dosages. Virtually all F_1 seeds formed after the 1.5- and 5-krad treatments were normal in appearance, whereas no viable seeds were obtained from the higher dosages. No seeds formed after pollinating with pollen that received the 15-krad treatment, while four small seeds were formed after pollinating with pollen that received the 30-krad treatment, but all were motes. Germination percentages for 1.5- and 5-krad WWRH F_1 seeds were high, 25/25 for the former and 24/26 for the latter. The two ungerminated 5-krad RH F_1 seeds were off-type, one was hollow and one was small, but other seeds were normal in appearance.

The preliminary genotypic evaluation was conducted on 44 WWRH F_1 plants, 22 randomly selected from each treatment of 1.5 and 5 krad, respectively. The plants were genotyped with 33 SSR markers from one linkage group and four chromosomes, of which eight, six, seven, six, and six markers were previously mapped to LG 9 and chromosomes 10, 12, 18, and 26, respectively (J. Z. Yu and R. J. Kohel, unpublished data). The incidence of deletions was considerably higher and the types of deletions were more diverse among 5-krad than among 1.5-krad WWRH plants. Among the 22 WWRHs from 1.5-krad γ-ray treatment, only 2 (9.1%) were identified as deletion lines. One plant lacked all six chromosome 12 markers, which indicates possible monosomy, and the other lacked just a terminal marker from the end of chromosome 10, most likely due to a single break and loss of a terminal segment (Figure 2). Marker retention frequency among the 1.5-krad plants was thus quite high, from 95.5 to 100% for individual loci, and averaged 98.4%. In contrast, among the 22 WWRHs from the 5-krad γ-ray treatment, 11 (50%) lacked one or more of the markers from LG 9 and/or chromosomes 10, 12, and 26, but no deletion lines observed involved chromosome 18. Four different types of deletions were detected (Figure 2). These included deletions of marker(s) at one end of a linkage group (one-end deletion, *e.g.*, from a single break), deletions of markers at both ends of a linkage group (two-end deletion, *e.g.*, from two breaks in opposite arms), deletions of interstitial marker(s) only [internal deletion, *e.g.*, from two breaks within an arm, followed by fusion or translocation(s)], and a more complex pattern (one-end plus internal deletion, *e.g.*, from three breaks, one leading to a terminal deletion and the other two occurring within an arm, leading to an interstitial deletion). For individual marker loci, retention frequency ranged from 77 to 100%, and the average across all loci was 93%. These findings strongly suggested that the 5-krad γ-ray treatment would be far more efficient than the 1.5-krad γ-ray

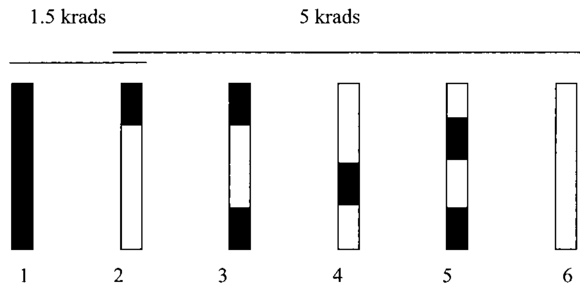

Figure 2.—Deletion classes observed in 1.5- and 5-krad panels. Each long bar represents a chromosome or linkage group. Each short solid bar represents a location where a chromosomal segment was deleted from the corresponding chromosome or linkage group. 1, whole deletion; 2, one-end deletion; 3, two-end deletion; 4, internal deletion; 5, one-end plus internal deletion; and 6, no deletion.

treatment for producing a WWRH panel and that the optimal radiation treatment dosage might be established between 5 and 15 krad of γ-rays.

SSR analysis of LG 9: Ninety-one additional WWRH seeds from 5-krad pollen irradiation were germinated, from which 79 additional WWRH F_1 plants were generated. Of the other 12 seeds, 9 were hollow (lacked a developed embryo), and 3 underwent radical elongation but died after initial seedling development. The 79 plants were combined with the pilot-screening population of 22 5-krad WWRH plants to construct a 5-krad WWRH panel of 101 individuals. LG 9 was chosen to evaluate the WWRH panel because eight available SSR markers (Table 1) were relatively evenly distributed along the linkage group (Figure 3; J. Z. Yu and R. J. Kohel, unpublished data). Two of them, BNL0625 and BNL2805, cosegregated in the linkage map population (http://demeter.bio.bnl.gov/acecot.html; J. Z. Yu and R. J. Kohel, unpublished data).

To characterize the deletion patterns, LG 9 was arbitrarily separated into eight parts in the dissection map in Figure 3. Each part was represented by one of the eight genotyped SSR markers in LG 9 regardless of their distances along the linkage group. Using the eight LG 9 SSR markers, 20 of the 101 WWRH plants were identified as deletion lines with 10 different deletion genotypes (Figure 3; Table 2). Individual deletion genotypes were observed in up to five WWRH individuals (Figure 3; Table 2). For individual SSR marker loci, the retention frequencies ranged from 87 to 94% with an average of 89.5% (Table 3). Chi-square tests indicated that marker retention frequencies did not depart significantly from the hypothesis that marker retention was independent of marker location on LG 9 (Table 3).

Whereas SSR markers BNL0625 and BNL2805 cosegregated in the traditional linkage mapping population, they were separated in WWRH plants GH6550 and GH6707. At least three breaks would have been required to form GH6550 and GH6707 genotypes (Table 2) if the natural order of loci along LG 9 were BNL2632-BNL0625-BNL2805-BNL3592. Alternatively, if the natural order were BNL2632-BNL2805-BNL0625-BNL3592, one break would have sufficed to form each of the GH6550 and GH6707 genotypes in Table 2 and Figure 3. Of 101 irradiated gametes represented in the WWRH panel, the likelihood of 2 gametes each with one break between BNL2805-BNL0625 would be much higher than the likelihood of 2 gametes each with three breaks between BNL2632-BNL0625, BNL0625-BNL2805, and BNL2805-BNL3592. Accordingly, the suggested locus order of BNL0625 and BNL2805 in LG 9 is BNL2632-BNL2805-BNL0625-BNL3592 (Figure 3). This order was

TABLE 1

Eight SSR markers of linkage group 9 tested in WWRH lines

Locus	Primers	Product size (bp) in TM-1
BNL0625	AGAGAGGGGGGAAAAGTTC GCCAGGCATGGTTTCTATGT	250
BNL0836	ATCTTGTTGATTTTCTGACTACAGG CAGACATTCCCCTTCCTTGA	190
BNL1066	ACATTTCCACCCAAGTCCAA ACTCTATGCCGCCTCTCGTA	130
BNL2632	CGTGTCTCCAGACCAACAAA GGGAGTTGAAGCCGACATAA	250
BNL2805	AGTTTGGAATTACAATAAATGTACTCG CCAAGGTCGGTCGGTTACTA	240
BNL3254	CACACAGTGTCCTTTGGGTG AGCCTCAAAGGCCAAAAGTT	130
BNL3592	GTTCTAGTCTCTTTCTTTTATGGGC TTGATTGAGATGCCAATGGA	200
BNL4094	ATGCTGCGGAGTCGATATC AAATTGATTTCATGCCGGAG	170

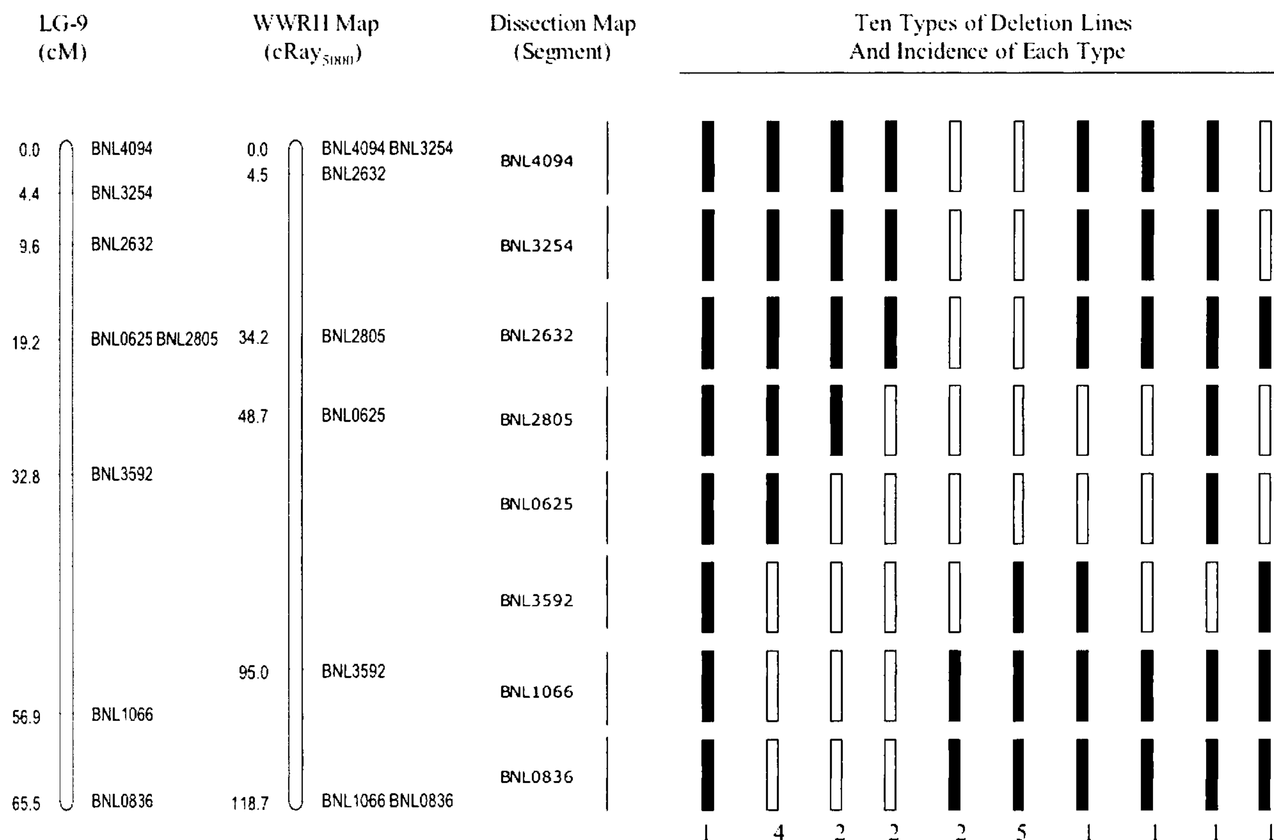

Figure 3.—Molecular dissection of LG 9 showing types of WWRH deletion lines based on eight LG 9 loci. Of 101 WWRHs resulting from 5-krad γ-ray pollen treatments, 20 were identified as lacking one or more LG 9 markers. The 20 deletion lines were grouped into 10 different types according to their pattern of SSR marker retention. The number of individuals in each type is listed below the genotype pattern. Leftmost is a traditional linkage map of LG 9, and immediately to its right is a WWRH map on LG 9, based on 93 WWRHs in the 5-krad mapping panel. Whereas SSR markers BNL0625 and BNL2805 cosegregated in the linkage mapping population, they were resolved in the WWRH panel (see text). They are depicted here according to a dissection map in which LG 9 is arbitrarily dissected into eight parts, each represented by one SSR marker. Solid bar, deleted chromosomal segment; open bar, undeleted chromosomal segment.

deduced on the basis of probability and minimum obligated breakage (Figure 3) and was supported by the maximum-likelihood RHMAP analysis with general retention probability model (Cox *et al.* 1990; Boehnke *et al.* 1995) using the 5-krad WWRH mapping panel (see below).

RHMAP analysis: For genotyping convenience on a 96-well apparatus that included both parents and a normal F_1, 93 WWRHs were randomly selected from the 101 5-krad WWRHs to form the 5-krad WWRH mapping panel. Good quality genotypic data were employed to run two-point RHMAP analyses. We used 102 SSR markers to genotype 93 WWRH plants. Fewer than 8 WWRH genotypes were uncertain for any marker. Syntenic groups were identified at three LOD score levels (LOD = 4, 6, and 8, respectively), as listed in Table 4. Fifty-two of 102 SSR markers were found to be syntenic with one or more SSR markers at LOD 4. In some cases, one linkage group was separated into different syntenic groups in the WWRH map. For example, LG 9 was separated into two syntenic groups. On the other hand, parts of different linkage groups were mapped by WWRH analysis into a single syntenic group, *e.g.*, part of LG 13 and part of LG 9 were mapped into the same syntenic group. No markers known to be located in different chromosomes were mapped into same syntenic group. However, one linkage group, LG 11, was found by WWRH analysis to be associated with two chromosomes, chromosome 1 and chromosome 9 (see next section).

Integrative mapping and cytogenetic stock analysis: According to our results, LG 9 and LG 13 involve markers from the same chromosome because several markers from each linkage group were mapped into the same syntenic group at LOD score 8 in our 5-krad WWRH panel. Cytogenetic aneuploid stocks were employed to identify the chromosomes associated with those two linkage groups and to test the WWRH-based deduction regarding their synteny. Although the collection of cotton hypoaneuploid cytogenetic stocks is still incomplete, we screened interspecific monosomic chromosome substitution stocks for chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 23, and 25 with SSR markers BNL1066

TABLE 2

Genotypes of WWRHs with deletion(s) in linkage group 9

RH lines	Markers							
	BNL4094	BNL3254	BNL2632	BNL0625	BNL2805	BNL3592	BNL1066	BNL0836
GH6541	+	?	+	+	?	−	−	−
GH6545	−	−	−	−	−	+	+	+
GH6550	−	−	−	+	−	+	+	+
GH6552	−	−	−	−	−	+	+	+
GH6556	+	+	+	+	+	−	−	−
GH6557	−	−	−	−	−	+	+	+
GH6558	−	−	−	+	+	−	−	−
GH6663	−	−	−	−	−	+	+	+
GH6678	+	+	+	+	+	+	−	−
GH6691	−	−	−	+	+	+	+	+
GH6692	−	−	−	−	−	−	−	−
GH6693	?	+	+	+	+	+	−	−
GH6699	+	+	+	+	+	−	−	−
GH6670	+	+	+	+	+	−	−	−
GH6702	+	+	+	+	+	−	−	−
GH6707	−	−	−	+	−	+	+	+
GH6713	−	−	−	+	+	+	?	+
GH6722	+	+	−	+	+	−	−	−
GH6724	−	−	−	−	−	+	−	−
GH6725	−	?	−	+	+	+	−	−

(−) Deleted for correspondent SSR marker; (+) heterozygous for correspondent SSR marker, *i.e.*, undeleted; (?) undecided genotype.

from LG 9 and BNL3442 from LG 13. Differential absence of both markers from H17 indicated that both markers are associated with chromosome 17 and that chromosome 17 harbors both LG 9 and LG 13 (Figure 4, A and B). Further analyses were conducted with the monotelodisomic "Te17sh," which contains a normal *G. barbadense* chromosome 17 and a *G. hirsutum* telosome for the short arm of chromosome 17 (deficient for all or most of the long arm). The presence of both markers indicated that this telosome contains both loci and most or all of LG 9 and LG 13 (Figure 5, A and B). A WWRH map was generated by concomitantly analyzing LG 9 and LG 13 loci with RHMAP maximum-likelihood analysis under the general retention model (Figure 6). Separation of BNL0625 and BNL2805 was confirmed and the distance between them was estimated to be 14.5 $cRay_{5krad}$ (Figure 6). The total WWRH map length of LG 9 was 118.7 $cRay_{5krad}$. The marker distance correlation coefficient between the traditional linkage map (J. Z. Yu and R. J. Kohel, unpublished data) and the WWRH map was 0.49. It was noted that the distance among markers from LG 13 was zero in the WWRH map. Ac-

TABLE 3

Linkage group 9 SSR marker retention frequencies and χ^2 test

Locus	No. typed (heterozygotes:deletions)[a]	Retention frequency
BNL0625	100 (94:6)	0.9400
BNL0836	99 (87:12)	0.8788
BNL1066	92 (80:12)	0.8696
BNL2632	100 (87:13)	0.8700
BNL2805	99 (91:8)	0.9192
BNL3254	95 (84:11)	0.8842
BNL3592	99 (91:8)	0.9192
BNL4094	99 (87:12)	0.8788
Average		0.8950
χ^2		5.37 ($\chi^2_{0.05}$ = 14.07; d.f. = 7)

[a] Number of WWRH lines unambiguously scored as deletion and nondeletion lines, followed by number of nondeletion lines and number of deletion lines in parentheses.

TABLE 4

Linkage groups detected by two-point RHMAP analyses at three LOD score levels

LOD score	SSR markers found to be syntenic
4	1604, 3065
	1667(lg11), 3888(lg11)
	2448(lg24), 3992(lg24)
	2553(lg17), 3646(lg17)
	2570(ch20), 3838(ch20)
	2646, 4082(lg11)
	2986(lg20), 3008(lg20)
	3103(ch25), 3264(ch25)
	836(lg9), 1066(lg9), 3592(lg9)
	1672, 3140(lg21), 3511(lg21)
	1705(lg26), 3449(lg26), 3976(lg26)
	2847(ch9), 3779(ch9), 4053(lg11)
	3255(ch5), 3474, 3792(g12)
	1317, 1350, 3345(lg11), 3902(lg11)
	1161(ch10), 1665(ch10), 2960(ch10), 3563(ch10), 3895(ch10)
	625(lg9), 1034(lg13), 1151(lg13), 1404(lg13), 1681(lg13), 2632(lg9), 2805(lg9), 3254(lg9), 3411(lg13), 3431(lg13), 3442(lg13), 4094(lg9)
6	1667, 3888
	3449, 3976
	1350, 3345, 3902
	836, 1066, 3592
	1161, 1665, 2960, 3563, 3895
	625, 1034, 1151, 1404, 1681, 2632, 2805, 3254, 3411, 3431, 3442, 4094
8	836, 1066
	1667, 3888
	1161,1665, 2960, 3563, 3895
	1034, 1151, 1404, 1681, 2632, 2805, 3254, 3411, 3431, 3442, 4094

The prefix BNL was omitted from the identification of each SSR marker. Marker locations if available in traditional linkage map are listed in parentheses at LOD 4. lg, linkage group; ch, chromosome.

cording to genotyping data, the average marker retention frequency for those markers on LG 13 was 92.4% with a range from 90.0 to 96.5% calculated from 93 WWRHs, whereas markers from LG 9 had an average of 89.5% with a range from 87.0 to 94.0% calculated from 101 WWRHs (Table 3). When all markers from both LG 9 and LG 13 were used, the chi-square test indicated that marker retention frequency did not differ significantly from homogeneity among SSR markers of the newly combined linkage group ($\chi^2 = 0.08 < \chi^2_{0.05} = 31.32$, d.f. = 14). However, we found significant differences in deletion types. For markers on LG 13, all 13 deletions (in 93 WWRHs) were of 1 deletion type (if not considering uncertain genotypes), whereas for LG

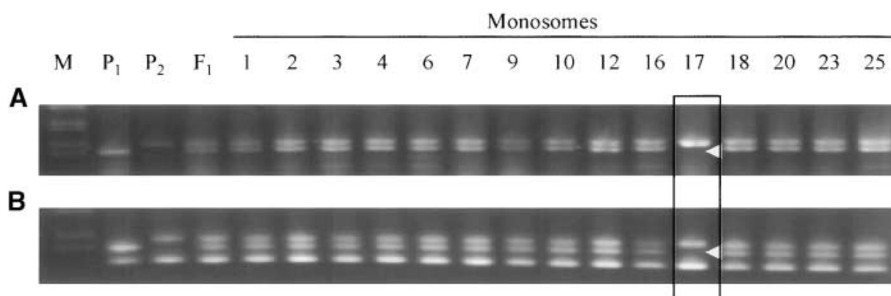

Figure 4.—Cytogenetic confirmation of the assignment of LG 9 and LG 13 to chromosome 17 using available monosomic chromosome substitution lines in cotton. Lanes 1–19 are sequentially as follows: molecular ladder (M); TM-1 (P_1); 3-79 (P_2); F_1 (3-79 × TM-1); and interspecific substitution stocks monosomic for chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 23, and 25, respectively. (A) Gel picture for marker BNL1066 from LG 9. (B) Gel picture for marker BNL3442 from LG 13. The monosomic substitution line for chromosome 17 is boxed, and the missing bands are denoted by arrowheads for both markers.

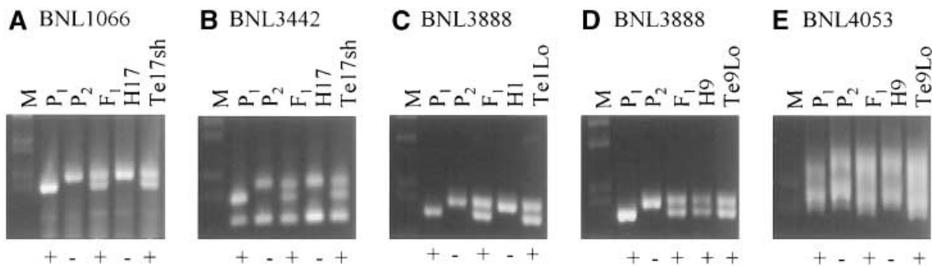

Figure 5.—Cytogenetic confirmation, using corresponding monosomes and ditelosomes, of the assignments of LG 9 and LG 13 to the chromosome 17 short arm (A and B), LG 11 to chromosome 1 (C) rather than to chromosome 9 (D), and marker BNL4053 on chromosome 9 long arm (E). Lanes 1–4 of A–E are the molecular ladder (M), TM-1 (P_1), 3-79 (P_2), and F_1 (3-79 × TM-1), respectively. Lane 5, monosomic substitution lines for chromosome 17 in A and B, chromosome 1 in C, and chromosome 9 in D and E, respectively. Lane 6, monotelodisomic lines Te17sh in A and B, Te1Lo in C, and Te9Lo in E and F, respectively. sh, short arm; Lo, long arm. Plus indicates target band present; minus indicates target band absent (see text).

9, there were 10 deletion types among 20 deletions in 101 WWRHs.

RHMAP analysis indicated that marker BNL4053 previously assigned to LG 11 (J. Z. Yu and R. J. Kohel, unpublished data) was syntenic to markers BNL3779 and BNL2847 from chromosome 9 at LOD 4. Cytogenetic analysis with interspecific F_1 hybrid stocks monosomic for chromosome 9 (H9) and monotelodisomic for chromosome 9 long arm (Te9Lo) indicated that BNL-4053 was on the long arm of chromosome 9 (Figure 5E). However, other markers from LG 11 could not be assigned to chromosome 9 according to additional cytogenetic analyses. For example, marker BNL3888 was actually associated with chromosome 1 (Figure 5C) rather than with chromosome 9 (Figure 5D). Examining LG 11, it was found that marker BNL4053 was mapped at one end of the linkage group, and the distance between it and the next marker BNL2921 was 44.4 cM at a LOD score of 3.46 (J. Z. Yu and R. J. Kohel, unpublished data). Thus the evidence for linkage to LG 11 was marginal. The results indicate that the WWRH mapping method correctly located marker BNL4053 into chromosome 9 and it corrected the false positive linkage in traditional linkage mapping.

DISCUSSION

WWRH mapping: The results indicate that WWRH mapping will provide a useful complement to linkage mapping of cotton and perhaps other plants. RH and WGRH methods have figured prominently in human and animal genomics (McCarthy 1996; Womack *et al.* 1997). In lieu of cell lines, technologies, and resources comparable to those in animals, plant researchers will likely devise a number of different and largely idiosyncratic approaches. Indeed, a chromosome-specific RH-like approach has been reported for maize, using oat-maize chromosome addition lines (Riera-Lizarazu *et al.* 1996, 2000). Extension of the approach to some other gramineous species seems feasible. A recent report of a WGRH panel for barley (Wardrop *et al.* 2002) suggests that an approach based on *in vitro* cell fusions may be feasible, but potential limitations and complications are numerous. These sorts of physical mapping tools are likely to significantly expedite development of genomics in some plants and its maturation in others.

The WWRH mapping method is essentially a modified form of WGRH mapping first reported by Walter *et al.* (1994). Like WGRH mapping, WWRH mapping requires just one panel and avoids the inefficiency of RH mapping that arises from the need for multiple mapping panels, usually one per chromosome or chromosome segment. By relying on *in vivo* rather than *in vitro* methods to rescue segmented genomes, the WWRH method remains technically and biologically simpler than the WGRH method. We used *G. barbadense* egg cells to rescue chromosomal segments of *G. hirsutum* sperm nuclei. The choice of *G. barbadense* as the rescue parent balanced the need for a high polymorphism level between the parents *vs.* the desire to avoid analytical complications and uncertainty that would result from potential genetic infidelity of plant *in vitro* culture (Evans 1989; Stelly *et al.* 1989). Furthermore, *G. hirsutum* × *G. barbadense* crosses have been used to establish the mapping populations and/or recombinant inbred lines for most cotton linkage mapping projects (Reinisch *et al.* 1994; Yu *et al.* 1998; Zhang *et al.* 2002; Lacape *et al.* 2003; Mei *et al.* 2004), and the particular genotypes employed herein have been used extensively as genetic and cytogenetic standards. The use of these species and genotypes to create the WWRH panel not only facilitated this evaluation of WWRH, but also will subsequently enable detailed assessment of linkage maps created with the same or closely related parents.

Pollen irradiation provided a relatively inexpensive means of segmentation. Except for hybrid clonal crops, most plant mapping populations are constructed by hybridization, followed by development of mapping populations at F_2, backcross, and/or advanced recombinant-inbred stages. In the WWRH method, the quasi-F_1 plants are well suited for direct use in mapping without further breeding. Panel and map development is thereby expedited, and all requirements for hybrid fertility are removed, which would otherwise constrain the choice of parents. The temporal efficacy of WWRH may have relatively greater significance for plant species with exten-

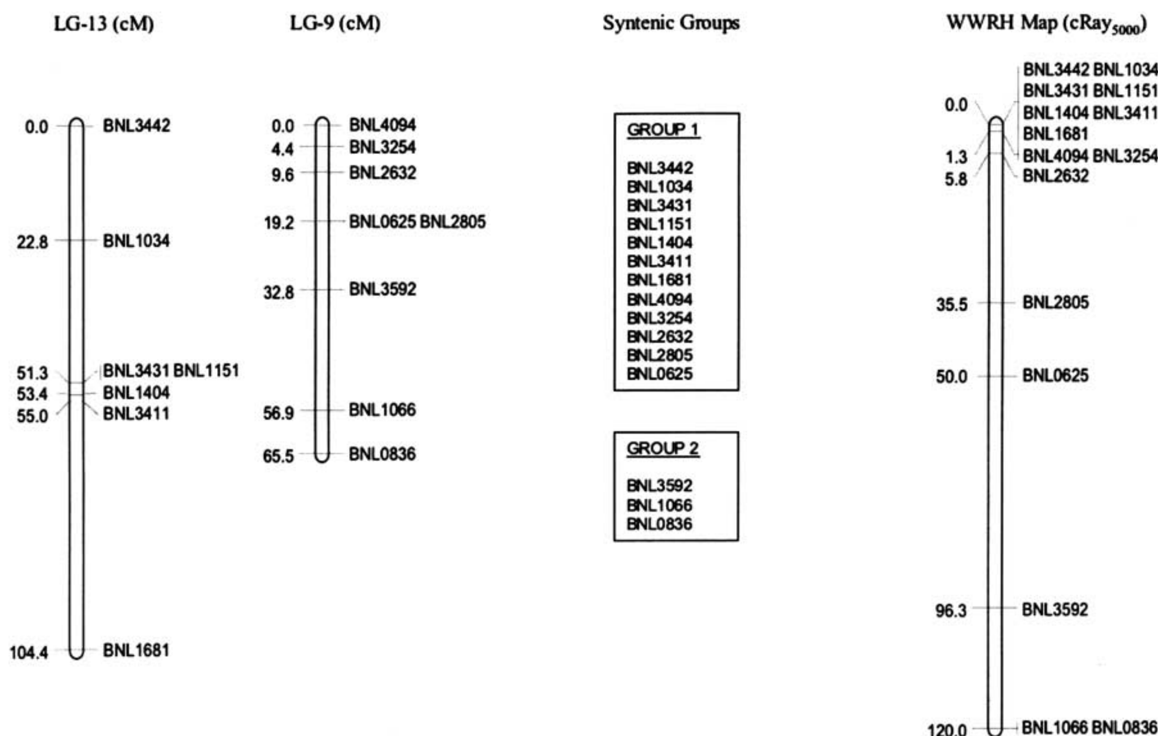

Figure 6.—Traditional linkage maps of LG 9 and LG 13 and the corresponding WWRH map. Middle, the syntenic groups at LOD 4 by two-point RHMAP analyses.

sive juvenility, especially as a means to expedite early steps in marker and map development and resolve uncertainties that arise from linkage analysis.

WWRH analysis might be advantageously applied to parents more diverse than those used herein to maximize the average level of marker polymorphism. WWRH panels can be constructed between any two parents that can form viable hybrids, without regard to their fertility. For example, use of a fertile synthetic A_2D_1 tetraploid rather than *G. barbadense* as recipient would enable WWRH mapping of relatively larger numbers of *G. hirsutum* markers, since more of them would be polymorphic between the parents. Moreover, *in vitro* ovule culture and embryo rescue might be used to increase polymorphism further by increasing the numbers of parental combinations that could be used to create WWRH panels. WGRH or RH mapping by *in vitro* cell culture methods offer potentially even higher polymorphism levels, but their potential usefulness may be very limited by chromosomal and/or genetic instability of *in vitro* plant cell cultures.

Derivation of each cotton WWRH panel member from a single-celled zygote avoided the genetic complications often entailed by somatic irradiation and *in vitro* culture of nonmeristematic plant cells. If irradiation had been applied to somatic tissue *in vivo* or *in vitro*, the initial product would have been a genomically heterogeneous cell population, development of which would have led to instability and chimerism of chromosome fragments. Although Kwock *et al.* (1998) reported all chromosomal segments could be retained without selection over 6 (zebrafish) and 25 (chicken) generations of subculture, respectively, rescued fragment instability in the host cell is a concern in traditional (WG)RH mapping approaches in human and animal species. The instability and somaclonal variation arising through nonmeristematic *in vitro* culture is a well-recognized phenomenon of many plant *in vitro* systems (Evans 1989), as are structural rearrangements (Stelly *et al.* 1989). The propensity of plant cells in nonmeristematic *in vitro* cultures to undergo chromosome aberration, mutation, and epigenetic modifications (Phillips *et al.* 1994) could jeopardize structural accuracy of RH and WGRH results.

Success of this WWRH effort was probably favored by the relatively high level of genetic redundancy that exists in cotton, but a more important factor may have been the developmental stage at which irradiation was applied. Relative to many species, cotton plants are quite tolerant of chromosomal and segmental aneuploidy, which can be attributed in large part to a high degree of genetic redundancy retained from its polyploid heritage (Endrizzi *et al.* 1984; Reinisch *et al.* 1994). In cotton, as in other angiosperms, tolerance of hypoaneuploidy is higher among the disomic sporophytes than among the haploid gametophytes, and sexual transmission of sporophytic hypoaneuploidy is much less frequent through microgametophytes (pollen) than through megagametophytes (Endrizzi *et al.* 1985; Birchler and Levin 1991). In constructing the WWRH panel, pollen-

mediated selection pressure against hypoaneuploidy was minimized by applying irradiation around anthesis, *i.e.*, after the first mitotic division of the microgametophyte. Aberrations and deficiencies were thereby induced independently in the generative and vegetative nuclei. According to existing dogma, pollen development and behavior are almost exclusively determined by the vegetative nucleus (Xu *et al.* 1999), and hypoaneuploidy of a generative cell or its descendent sperm cells generally would have little or no impact on pollen phenotype. In keeping with this view is the fact that gametophytic aneuploidy is more likely to be transmitted if it arises *de novo* during gametophyte development from a euploid spore rather than from an aneuploid spore that forms a uniformly aneuploid gametophyte. These biological principles are well exemplified by the cytogenetic manipulations that are used to produce maize hypoaneuploids, *i.e.*, mitotic nondisjunction in microgametophytes subjected to B-A translocation chromosome manipulations to recover segmental deficiencies (Beckett 1978) and in corn *rX-1* mutant megagametophytes to recover monosomic progeny (Helentjaris *et al.* 1986; Lin and Coe 1986; Simcox *et al.* 1987). In the WWRH method used here, a different set of induced aberrations and deficiencies was expectedly transmitted to each zygote, and the single-cell origin of the resulting WWRH embryo and plant tissue minimized heterogeneity and chimerism. Interestingly, chimerism was observed in the RH mapping of maize chromosome 9 where irradiation was carried out on seed (Riera-Lizarazu *et al.* 2000) rather than on pollen.

Marker retention frequency and randomness: In (WG)RH mapping of human and animal species, chromosome retention frequencies ~30% are considered efficient. The average marker retention frequency in maize RH mapping was 75–85% (Riera-Lizarazu *et al.* 2000). In this study, we observed retention frequencies from 87 to 94% for individual SSR markers in LG 9. The high retention frequencies and the lack of seed formation after higher dosages of pollen irradiation probably reflect that significant selection occurred during pollen germination, tube growth, and fertilization and, perhaps, early embryo development.

Dosage is a critically important feature of any irradiation-based segmentation mapping effort. On average, pollen with smaller and fewer deletions would expectedly be more viable and more competitive than pollen with larger and more numerous deletions. We observed that the 5-krad γ-ray treatment led to more chromosomal breakage, more deletion types, and lower chromosomal retention frequencies than the 1.5-krad γ-ray treatment. Riera-Lizarazu *et al.* (2000) also observed that marker retention frequency decreased nonsignificantly with increasing radiation dosage, whereas the number of retention patterns increased significantly. Results for animals have been mixed. After higher dosages, marker retention frequencies changed nonsignificantly for chicken (Kwock *et al.* 1998), but were higher for bovine (Womack *et al.* 1997; Rexroad *et al.* 2000) and horse (Kiguwa *et al.* 2000; Chowdhary *et al.* 2002). Our results suggest higher radiation dosages, *e.g.*, ~5- to 15-krad range might improve efficacy by further lowering the chromosomal retention frequency and, perhaps most importantly, increasing the number of retention patterns.

Radiation hybrid mapping software programs offer several models regarding nonrandom transmission or retention of chromosomal segments, so nonrandomness of retention, *per se*, does not preclude mapping. For example, the RHMAP program by Boehnke *et al.* (1995) offers four models: equal retention probability model, centromeric retention probability model, left-endpoint retention probability model, and general retention probability model. Although tests indicated that retention rates were similar among markers in LG 9 and LG 13, we subjected the data to RHMAP analysis under two probability models, one for equal retention and one for general retention, which allows retention probabilities to differ. The resulting orders of loci were identical, and the distances among markers were only slightly different (data not shown).

WWRH mapping as a complement to traditional linkage mapping: The results suggest that WWRH maps will generally provide higher resolution than linkage maps among loci in regions that undergo little recombination and/or from which the recovery of recombinant products is strongly reduced. In addition, WWRH will facilitate the detection of synteny and thus mapping of segments that flank extremely high-recombination regions. Ramifications could extend from linkage group identification and nomenclature to integrated mapping, cloning, functional genomics, proteomics, and bioinformatics. WWRH mapping will also provide a means to test linkage mapping results and to detect cryptic map distortions from structural genomic differences between the parents.

A common problem in linkage maps is the occurrence of multilocus bins unresolved by homologous recombination. Cotton metaphase I bivalent configurations reveal that chiasmata virtually never occur near centromeres and indicate that the intrachromosomal distribution of recombination is highly nonrandom in cotton, as it is in many if not all other higher eukaryotes (reviewed in Puchta and Hohn 1996; Copenhaver *et al.* 1998; Gerton *et al.* 2000; reviewed in Petes 2001; Yu *et al.* 2001). The large tracts of heterochromatin typically found in plant chromosome centric regions can collectively constitute much of a genome, but be unresolved by linkage mapping (Islam-Faridi *et al.* 2002). The use of large-insert genomic clones that contain linkage-mapped marker loci enables the use of integrative molecular cytogenetic analysis to delimit boundaries of low-recombination regions on linkage maps (Islam-Faridi *et al.* 2002), but internal definition remains prob-

lematic. The abundance of repetitive sequences in plant centric heterochromatin renders molecular cytogenetic probes from nearly all large-insert genomic clones ineffective for detection of individual linkage map loci (Hanson *et al.* 1995, 1998). Thus, an additional technique is needed for integrative mapping of these large tracts of centric region heterochromatin. The ability of WWRH mapping to resolve and order closely linked loci was exemplified by separation and ordering of LG 9 SSR markers BNL0625 and BNL2805. Once loci are ordered, whether by WWRH or linkage analysis, they can be used more effectively for molecular manipulations, *e.g.*, contig orientation and assembly. Since linkage mapping cannot resolve low-recombination regions, molecular cytogenetic, WWRH, or similarly capable physical mapping methods will be required to generate comprehensive genome maps of most plant species.

High-recombination regions of significant "length" pose a different problem for linkage mapping, because marker density within them must be high to detect linkages. Failure to do so, as is common during early phases of map development, can lead to excessive numbers of seemingly independent linkage groups. However, WWRH mapping and certain other physical mapping methods offer a generic ability to span high-recombination segments and thereby enable mapping projects to detect synteny between loci within and/or flanking such segments. Intraspecific meiotic configuration analysis and interspecific linkage analysis both indicate that the cotton genome map is 4500 cM or larger (Menzel *et al.* 1985; Stelly 1993; Reinisch *et al.* 1994), which is considerably longer than genomes of bread wheat (3791 cM), soybean (3159 cM), corn (1807 cM), rice (1530 cM), tomato (1472 cM), and barley (1279 cM; National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/mapview/). Moreover, cytological observations and linkage mapping results both indicate the genome is peppered with high-recombination regions. Hypoaneuploid interspecific F_1 hybrid cytogenetic stocks are quite effective for addressing problems the high-recombination regions create, but their collective coverage of the cotton genome is estimated at only 70–80% and complete coverage of individual chromosomes is provided only by monosomics, which are available for only ~60% of the chromosomes (Stelly 1993; D. M. Stelly, unpublished data). Thus, additional physical mapping methods are needed to detect synteny across high-recombination regions. For WWRH, this capability was exemplified by coalescence of the LG 9 and LG 13 of a traditional linkage map into one syntenic group. The cotton WWRH panel will expectedly enable various mapping projects to reduce their numbers of cotton linkage groups to the gametic chromosomal number (26).

Most of the cotton linkage mapping populations have been developed from interspecific hybrids of the two cultivated AD-genome ($n = 26$) species, *G. hirsutum* ($[AD]_1$ genome) and *G. barbadense* ($[AD]_2$ genome; Reinisch *et al.* 1994; Yu *et al.* 1998; Zhang *et al.* 2002; Lacape *et al.* 2003; Mei *et al.* 2004). The hybrids provide complementary traits of economic significance and offer higher rates of molecular marker polymorphism, which are low intraspecifically (Brubaker and Wendel 1994). Although these two species hybridize freely, subsequent generations are subject to intense natural selection. Their genomic structure is similar at a gross level, but has yet to be compared comprehensively in detail. Given that the likelihood of structural differences increases with genetic distance, it seems highly likely that cryptic effects of structural differences exist between mapping parents and that these would inadvertently influence linkage maps. Indeed, distortion of linkage maps by translocations and inversions has been demonstrated by computer simulation (Livingstone *et al.* 2000) and integrative mapping of oat (Wight *et al.* 2003). Distorted marker segregation and some clustering of markers has been observed in cotton linkage mapping populations of interspecific hybridization (Reinisch *et al.* 1994; Zhang *et al.* 2002; Mei *et al.* 2004). In fact, distorted marker segregation was also observed in an intraspecific *G. hirsutum* mapping population (Shappley *et al.* 1998). To screen genomes of mapping parents for structural differences and assess their impact on genome maps will require WWRH or some other form of integrated physical mapping.

WWRHs for chromosomal identification of markers and linkage groups: WWRH mapping is incapable of directly establishing chromosomal identity of unassigned markers and linkage groups, but it can do so indirectly by revealing their synteny to markers of known chromosomal identity. In this study, WWRH analysis differentiated between BNL4053 and other LG 11 markers and uniquely associated BNL4053 to chromosome 9 markers BNL3779 and BNL2847. The WWRH findings were supported by cytogenetic tests and by reexamination of the linkage data, which showed BNL4053 to be a terminal marker only weakly associated with the most proximal LG 11 locus. The results suggest that WWRHs can be used to chromosomally identify markers and linkage groups, as a complement to the cotton cytogenetic stocks in terms of both coverage and increased subchromosomal specificity.

Prospects: The results indicate that WWRH mapping offers a facile yet potent means to verify linkage maps, bin, order, and map loci in the cotton genome and that WWRH will be especially valuable for mapping regions that are very lowly and very highly recombinant. WWRH will enable researchers to reduce the numbers of linkage groups in their respective maps and help foster development of a common linkage group nomenclature. Besides improving basic information and communication, these improvements will facilitate integration of genomic resources. Analogous applications seem plausible

for other species, including those that are relatively recalcitrant to accurate linkage mapping due to genomic complexity, genome structural variability, apomixis, polysomy, hybridity, cultivar-specific interests, and/or long generation times. The importance of polyploidy *per se* to success of WWRH is unknown. While polyploidy would seem to favor its applicability genomewide, pollen irradiation has also been used effectively to recover segmental deletions in plants conventionally viewed as diploid, including Arabidopsis (Vizir *et al.* 1994).

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