Note

Completely Distinguishing Individual A-Genome Chromosomes and Their Karyotyping Analysis by Multiple Bacterial Artificial Chromosome–Fluorescence *in Situ* Hybridization

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ABSTRACT

Twenty bacterial artificial chromosome (BAC) clones that could produce bright signals and no or very low fluorescence *in situ* hybridization (FISH) background were identified from *Gossypium arboreum* cv. JLZM, and *G. hirsutum* accession (acc.) TM-1 and 0-613-2R. Combining with 45S and 5S rDNA, a 22-probe cocktail that could identify all 13 *G. arboreum* chromosomes simultaneously was developed. According to their homology with tetraploid cotton, the *G. arboreum* chromosomes were designated as A1–A13, and a standard karyotype analysis of *G. arboreum* was presented. These results demonstrated an application for multiple BAC–FISH in cotton cytogenetic studies and a technique to overcome the problem of simultaneous chromosome recognition in mitotic cotton cells.

COTTON is one of the most important natural fiber and edible oil crops in the world. Significant progress has been made in the development of genetic maps for the purpose of gene and quantitative trait loci (QTL) mapping (REINISCH *et al.* 1994; ULLOA *et al.* 2002; ZHANG *et al.* 2002; MEI *et al.* 2004; NGUYEN *et al.* 2004; RONG *et al.* 2004; SONG *et al.* 2005; HAN *et al.* 2006; GUO *et al.* 2007). However, due to the large number and small size of the chromosomes and especially the absence of suitable cytogenetic markers such as bands, chromosome identification has lagged significantly behind the development of linkage mapping. Although many genetic materials such as translocation, aneuploid lines, and the nomenclature for the tetraploid cotton chromosomes have been developed (BROWN 1980; ENDRIZZI *et al.* 1985), the routine and unambiguous identification of individual chromosomes, especially in mitotic cells, is almost impossible in cotton. The bacterial artificial chromosome–fluorescence *in situ* hybridization (BAC–FISH) technique has been developed and used in physical mapping (HANSON *et al.* 1995), and individual mitotic chromosomes were easily identified in

cotton (WANG *et al.* 2006). Then one set of chromosome-specific BACs was developed, and the comparison mapping between physical and genetic maps was conducted (WANG *et al.* 2007a,b). Therefore, BAC–FISH has shown a unique superiority for cotton chromosome identification and physical mapping.

The cotton genus *Gossypium* is composed of 45 diploid and 5 tetraploid species. Among them 4 are cultivated *Gossypium* species: *G. hirsutum* L. [$n = 2x = 26$, (AD)₁], *G. barbadense* L. [$n = 2x = 26$, (AD)₂], *G. arboreum* L. ($n = x = 13$, A₂), and *G. herbaceum* L. ($n = x = 13$, A₁). Tetraploid species (*G. hirsutum* L. and *G. barbadense* L.) dominate worldwide cotton production. *G. arboreum*, as an A-genome diploid cotton, has been domesticated and cultivated in China for almost 2000 years (XIANG and SHEN 1989). Due to some of its superior agronomic traits, such as disease and insect resistance, high fiber strength, and excellent plasticity, which upland cotton cultivars lack, *G. arboreum* is still planted and is used worldwide as a germplasm resource in present-day cotton breeding programs. *G. arboreum* is generally regarded as one of the best exemplars of the A-subgenome progenitors (ENDRIZZI *et al.* 1985; WENDEL *et al.* 1995). Therefore, the *G. arboreum* species is important for agricultural production and genomic and evolution research in cotton.

Here, we described the screening of one diploid cotton (*G. arboreum*) and two tetraploid cotton (*G.*

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FIGURE 1.—FISH results of a 22-probe cocktail hybridized to *G. arboreum* mitotic chromosomes simultaneously. The 5S rDNA and BAC 84C03 on chromosome A4 were labeled by both biotin and digoxigenin, thus the white or yellow signals on chromosomes A4 and A9 were found. The patterns of signals enable us to recognize each chromosome pair according to the homologous relationship with the A-subgenome chromosomes of *G. hirsutum*. Bar, 10 μ m.

hirsutum) BAC libraries to identify new chromosome-specific clones. Combining with previous BAC clones and 45S and 5S rDNA, a 22-probe cocktail was developed for the purpose of simultaneous chromosome identification and karyotyping in *G. arboreum*. This enabled us to identify all of the 13 *G. arboreum* chromosome pairs simultaneously. According to their homology with the A-subgenome (At) chromosomes in tetraploid cotton, the nomenclature and standard karyotype of *G. arboreum* chromosomes were developed.

MATERIALS AND METHODS

The BACs used in this study came from three genomic BAC libraries, of which two were constructed from the tetraploid cotton *G. hirsutum* accession (acc.) TM-1 (Y. HU and T. Z. ZHANG unpublished data), and *G. hirsutum* acc. 0-613-2R, a cytoplasm male fertility line (WENDEL *et al.* 1995; YIN *et al.* 2006), and A-genome *G. arboreum* cv. JLZM (Y. HU and T. Z. ZHANG unpublished data). The corresponding SSR markers used to screen the BAC clones were selected from the tetraploid map (GUO *et al.* 2007) and a new A-genome genetic map (MA *et al.* 2007). The library screening was conducted as described previously by WANG *et al.* (2006). The 45S and 5S rDNA derived from *Arabidopsis thaliana* were kindly supplied by X. E. WANG, Nanjing Agricultural University, China. All the BAC clones were initially evaluated in the FISH, and only BAC clones that could produce bright signals and no or very low background were selected as candidates for multiple FISH.

To reidentify their physical locations, all BAC candidates for which the corresponding genetic markers were derived from the same linkage group were hybridized simultaneously with the At-derived chromosome-specific BACs (WANG *et al.* 2007b) in FISH on both JLZM and TM-1 mitotic metaphase chromosomes. When different locations occurred, more At-derived chromosome-specific BACs were used to hybridize in the simultaneous FISH to find the new BAC locations. Root tip,

slide preparation, and single- and dual-color FISH have been described previously (WANG *et al.* 2006). For the 22-probe cocktail FISH, some modifications were made as follows: All the BAC DNA or rDNA were labeled by biotin and digoxigenin, detected by anti-digoxigenin-rhodamine (red) and avidin-fluorescein (green), and the white or yellow color was conducted by labeling the probe with biotin and digoxigenin. For the probe-cocktail mixture, 100 ng of each of the labeled probe DNA, 5 μ g Cot-1 DNA derived from TM-1 and JLZM, respectively, were mixed together, precipitated in ethanol, and then dissolved in 4 μ l distilled water plus 10 μ l formamide, 2 μ l 20 $\times$ SSC and 4 μ l 50% (w/v) dextran sulfate. After the probe mixture was denatured at 97 $^{\circ}$ for 10 min, it was annealed for 1 hr before being applied to the slide.

Following the posthybridization washes, chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Sigma, St. Louis). The slides were examined under an Olympus BX51 fluorescence microscope. Chromosome and FISH signal images were captured using an Evolution VF CCD camera (Media Cybernetics, Bethesda, MD) and merged using Image-Pro Express software. For karyotyping images, DAPI-stained chromosomes were measured using the same Image-Pro Express software. The arm ratio (average long arm/short arm ratio), total chromosome length (short arm + long arm), and relative chromosome length (length of the individual chromosome/total length of all chromosomes in the genome) were calculated for each of the 20 mitotic chromosomes.

RESULTS AND DISCUSSION

Cytogenetic studies rely on accurate and consistent chromosome identification, which is always a challenge in plant species with small chromosomes. Several *G. arboreum* karyotypes have been reported on the basis of chromosome identification by length (SKOVSTED 1933; SKOVSTED 1934; WU and CHEN 1984; GENNUR *et al.* 1988; SONG *et al.* 1991; WANG *et al.* 1997). However, the use of different materials and measurement variability

FIGURE 2.—BAC clones and their corresponding genetic markers of the 22 probes according to FISH signal patterns in Figure 1. Chromosomes were depicted with the shorter arm in the top position according to FISHed individual chromosome (right). The genetic markers and their corresponding BAC clones were indicated on the left and right of the chromosome diagrams, respectively. Single asterisk and double asterisks indicated BAC clones derived from the *G. arboreum* and *G. hirsutum* in this research, respectively, and the rest were *G. hirsutum* BAC clones but developed previously (WANG *et al.* 2007b).

between laboratories can potentially result in the misidentification of chromosomes. Consequently, significant discrepancies exist among the previously published karyotypes. More importantly, as one of the ancestor species of tetraploid, none of these *G. arboreum* karyotypes are integrated with the tetraploid or genetic linkage maps. To remove these limitations, we isolated a set of 20 BAC clones from A and At genomes using genetically mapped SSR markers. Combining with the 45S and 5S rDNA, this 22-probe cocktail not only allowed the correct identification of all 26 mitotic metaphase chromosomes of *G. arboreum* cv. JLZM, but also enabled us to completely integrate our karyotype with the genetic linkage map of *G. arboreum* (Figures 1 and 2). Moreover, the identification of BAC clones, which contain no or less repetitive DNA and generate bright and low or no background, is critical for the success of

BAC-FISH. BAC-FISH has not been widely used in cotton cytogenetic studies due to the high percentage of repetitive sequences. In this study, we identified twenty BACs from more than fifty BACs, and successfully demonstrated the use of multiple BAC-FISH for cytogenetic research in cotton.

Among this 22-probe cocktail, six BAC probes were derived from the A-genome species *G. arboreum* cv. JLZM BAC clones, and all the others were from the tetraploid TM-1 or 0-613-2R clones, including nine previously identified chromosome-specific BACs (WANG *et al.* 2007a,b; WANG *et al.* 2006). Through the cohybridization with one set of At chromosome-specific BACs, the physical locations of diploid BACs were identified and the chromosomal nomenclature of *G. arboreum* was aligned with the tetraploid cotton, in that the JLZM chromosomes were designated as A1–A13 according to the At chromosomes (Figures 1 and 2). Therefore, our nomenclature system for *G. arboreum* was integrated with the At chromosomes. The linkage groups of the *G. arboreum* genetic map could also be identified according to the A- and At-derived BACs and corresponding SSR markers. The uniform nomenclature between the diploid A- and tetraploid At-genomes in cotton will facilitate chromosome identification, ease communication between different research groups, and facilitate data usage and comparisons between A- and At-genome maps.

On the basis of the identification of metaphase chromosomes, a FISH-based karyotype of *G. arboreum* JLZM was developed (Table 1). Our results show that the lengths of the mitotic metaphase chromosomes in the current numbering system are not in descending order. The current descending order of the metaphase lengths is A5 (including the rDNA section), A7 (including the rDNA section), A3, A4, A6, A1, A12, A2, A10, A8, A13, A11, and A9. All the chromosomes could be grouped into two classes according to their total lengths. Seven chromosomes (A1, A3, A4, A5, A6, A7, and A12) are the longer chromosomes and the other six (A2, A8, A9, A10, A11, and A13) are the shorter ones. The longest chromosome was chromosome A5, which had an absolute length of 3.79 μm and a relative length of 8.85%. The shortest chromosome was A9, which had an absolute length of 2.80 μm and a relative length of 6.55%. None of the chromosomes were exceptionally long or short, and all of them were metacentric except for chromosome A12, which was the only pair of mid-sized but sub-metacentric chromosomes.

Through the exceptional appearance of secondary constriction on the chromosome, some researchers reported finding one or two nucleolar organizer regions (NORs) in different *G. arboreum* species using the classical dyeing method (EDWARDS 1977; WU and CHEN 1984; GENNUR *et al.* 1988; SONG *et al.* 1991). Two major NOR loci were found using rDNA FISH in different *G. arboreum* species (HANSON *et al.* 1996; WANG *et al.* 2001; BIE *et al.* 2004). At the same time, two major NOR loci

TABLE 1
Length and arm ratio of the mitotic chromosomes in JLZM

Chromosome	Total length (μm)	Relative length ^a (%)	DNA content ^b (Mb)	Location of NOR	Arm ratio ^c
A1	3.44 $\pm$ 0.35	8.02 $\pm$ 0.81	94.0 $\pm$ 13.91	—	1.30 $\pm$ 0.15
A2	3.04 $\pm$ 0.26	7.10 $\pm$ 0.61	83.2 $\pm$ 10.57	—	1.25 $\pm$ 0.22
A3	3.60 $\pm$ 0.34	8.40 $\pm$ 0.79	98.4 $\pm$ 13.60	—	1.06 $\pm$ 0.06
A4	3.59 $\pm$ 0.48	8.37 $\pm$ 1.11	98.0 $\pm$ 19.07	—	1.20 $\pm$ 0.16
A5	3.79 $\pm$ 0.39	8.85 $\pm$ 0.92	103.6 $\pm$ 15.73	Short arm	1.19 $\pm$ 0.12
A6	3.57 $\pm$ 0.48	8.33 $\pm$ 1.12	97.6 $\pm$ 19.31	—	1.12 $\pm$ 0.06
A7	3.69 $\pm$ 0.47	8.61 $\pm$ 1.09	100.8 $\pm$ 18.74	Short arm	1.32 $\pm$ 0.24
A8	3.01 $\pm$ 0.31	7.03 $\pm$ 0.72	82.3 $\pm$ 12.39	—	1.13 $\pm$ 0.10
A9	2.80 $\pm$ 0.31	6.55 $\pm$ 0.71	76.6 $\pm$ 12.21	—	1.29 $\pm$ 0.19
A10	3.02 $\pm$ 0.31	7.04 $\pm$ 0.71	82.4 $\pm$ 12.19	—	1.20 $\pm$ 0.14
A11	2.91 $\pm$ 0.32	6.79 $\pm$ 0.74	79.5 $\pm$ 12.82	—	1.37 $\pm$ 0.21
A12	3.41 $\pm$ 0.38	7.96 $\pm$ 0.89	93.3 $\pm$ 15.17	—	1.86 $\pm$ 0.23
A13	2.97 $\pm$ 0.32	6.94 $\pm$ 0.74	81.2 $\pm$ 12.71	—	1.27 $\pm$ 0.28

^aRelative length, chromosome length/genome length.

^bEstimation of the DNA content of each chromosome is based on the assumption that the haploid genome size of *G. arboreum* is 1171 Mb (HENDRIX and STEWART 2005); thus, the DNA content will be relative length $\times$ estimated genome size, *i.e.*, 1171 Mbp.

^cArm ratio, length of the long arm/the length of the short arm.

were also found in *G. herbaceum*, a diploid species highly homologous with *G. arboreum*. Although the different *G. arboreum* species might contain different numbers of NORs resulting from long-term evolution (STEBBINS 1952; GENNUR *et al.* 1988), we expected the rDNA FISH technique to result in more accurate results.

We also located two major NORs in this study, using the 45S rDNA cohybridization technique with some chromosome-specific BAC clones in FISH. One NOR was located near the end in the short arm of chromosome A5 and another was located at the end in the short arm of chromosome A7. A very minor 45S rDNA signal was also found and located on the chromosome A9 when using the single 45S rDNA as a probe in FISH. Its signal locus had some overlap with that of the 5S rDNA (also located on chromosome A9) and was too weak to be detected in multiple FISH when we adjusted the photo contrast. HANSON *et al.* (1996) found two major NORs in the A-subgenome of *G. hirsutum*, then they found another major NOR and located all three NORs on chromosomes At5, At7, and At9 (JI *et al.* 1999). All of these results support our findings and indicate that *G. arboreum* was one of the ancestors of *G. hirsutum*.

It is widely accepted that *G. arboreum* or *G. herbaceum* (A-genome species) and *G. raimondii* (D-genome species), the ancestors of the A- and D-subgenomes of tetraploid cotton, respectively, were derived from the same ancestor 6–11 million years ago (WENDEL 1989). Cytogenetic studies have revealed that the A-genome species differed from each other by a single translocation, whereas the A-subgenome differed from A, D, and Dt by two reciprocal translocations (BROWN and MENZEL 1950; GERSTEL 1953; MENZEL and BROWN 1954; ENDRIZZI *et al.* 1985). The molecular data derived from tetraploid cotton also showed two translocations between chromosomes

At2 and At3, and At4 and At5 (BRUBAKER *et al.* 1999; RONG *et al.* 2004; DESAI *et al.* 2006; GUO *et al.* 2007). Our FISH results confirmed these findings. Two BAC translocations involving two chromosome pairs of A2 and A3 and A4 and A5 between *G. arboreum* and the A-subgenome of *G. hirsutum* were found. As shown in Figure 3, one BAC 64F22 that located on At2 of *G. hirsutum* was located on chromosome A3 of *G. arboreum*. It appears that the chromosomal segment containing the BAC 64F22 was involved in a translocation between chromosomes A3 and At2. Another BAC translocation was found between the chromosomes At4 and A5 shown in Figure 3. These results are consistent with previous cytological (BROWN and MENZEL 1950; MENZEL and BROWN 1954) and molecular (BRUBAKER *et al.* 1999; RONG *et al.* 2004) data. But two reciprocal translocations were found in their research. Unfortunately, because of the few BACs identified here, no translocation BACs were found on the other two pairs of chromosomes, A2 and At3 and A4 and At5. Therefore, we could not identify these two cases as reciprocal translocations, but with effort, we believe complementary results will be achieved. Moreover, although only a few translocation BACs were found here, the large size of the DNA segment represented should be expected to provide more powerful translocation detection.

Additionally, the results did not present an integrated “cytogenomic” map between physical and genetic maps, mainly because of the few JLZM BACs available. However, relationships were established between the 13 chromosomes of *G. arboreum* and linkage groups of A-subgenome chromosomes of *G. hirsutum*, and, no doubt, these data will facilitate linkage map construction and chromosomal assignments in *G. arboreum* and assist in the isolation and construction of a BAC-by-BAC

FIGURE 3.—Two BAC translocations involving two chromosome pairs of At2 and A3 and At4 and A5 between *G. arboreum* and A-subgenome of *G. hirsutum*. The colored circles represent signals of BAC clones linked with BAC clones and corresponding markers. The translocation BAC clones were linked between chromosome pairs of At2 and A3 and At4 and A5.

physical map and even in the sequencing of the individual chromosomes.

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