Fluorescent in situ hybridization of a bacterial artificial chromosome

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Abstract: Fluorescent in situ hybridization (FISH) of a 130 kilobase cotton (*Gossypium hirsutum* L.) bacterial artificial chromosome (BAC) clone containing a high proportion of single-copy DNA produced a large pair of FISH signals on the distal end of the long arm of a pair of chromosomes of the D-genome species *G. raimondii* Ulbr. and produced a fainter pair of signals on a small submetacentric pair of chromosomes of the A-genome species *G. herbaceum* L. The signals were syntenic with a nucleolar organizer region in *G. raimondii* and *G. herbaceum*. Signal pairs were easily recognized in interphase and metaphase cells either with or without suppression of repetitive sequences with unlabeled *G. hirsutum* C_0t-1 DNA. High quality FISH results were consistently obtained and image analysis was not required for viewing or photography. Results indicate that FISH of BAC clones is an excellent tool for the establishment of new molecular cytogenetic markers in plants and will likely prove instrumental in the development of useful physical maps for many economically important crop species.

Key words: bacterial artificial chromosome, BAC, *Gossypium*, in situ hybridization, physical mapping.

Résumé : L'hybridation in situ à fluorescence (FISH) a été réalisée avec un chromosome bactérien artificiel (BAC) de 130 kb contenant de l'ADN du cotonnier (*Gossypium hirsutum* L.). Ce clone, contenant une forte proportion d'ADN à simple copie, a produit une paire de forts signaux FISH à l'extrémité terminale du bras long d'une paire de chromosomes de l'espèce à génome D *G. raimondii* (Ulbr.) ainsi qu'une paire de signaux plus faibles sur une petite paire de chromosomes submétacentriques de l'espèce à génome A *G. herbaceum* (L.). Ces signaux étaient localisés sur des chromosomes montrant également une région d'organisation nucléolaire chez le *G. raimondii* et le *G. herbaceum*. Les paires de signaux étaient facilement reconnaissables dans les cellules en interphase et en métaphase, avec ou sans suppression des séquences répétées avec l'ADN C_0t-1 non-marqué du *G. hirsutum*. Des résultats de haute qualité ont été obtenus de façon reproductible et l'analyse d'images n'a pas été requise pour fins d'examen ou de photographie. Les résultats indiquent que la méthode FISH avec des clones BAC est un excellent outil dans le but d'établir de nouveaux marqueurs moléculaires cytogénétiques chez les plantes. Cette méthode pourrait aussi contribuer au développement de cartes physiques chez de nombreuses espèces importantes sur le plan économique.

Mots clés : chromosomes bactériens artificiels, BAC, *Gossypium*, hybridation in situ, cartographie physique.

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Introduction

In situ hybridization (ISH) is a powerful analytical tool, one important application of which is genomic mapping. It is especially valuable for physical mapping, including relating recombination-based maps to physical maps based on chromosome structures, banding, physical distances, and molecular cytogenetic loci. In situ hybridization also can be used to map ISH sites relative to various cytogenetic aberrations (such as deletions and translocation breakpoints), thus promoting development of an integrated map that spans recombinational distances, physical distances,

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Fig. 1. CHEF gel of *G. hirsutum* BAC clones stained with ethidium bromide. BAC DNA was isolated by alkaline lysis plasmid minipreparations. Inserts were excised with *Not*I restriction endonuclease digestion prior to electrophoresis. The size of the BAC 1928 insert (white arrow) was estimated to be approximately 130 kb; lambda marker shown at left (white dots).

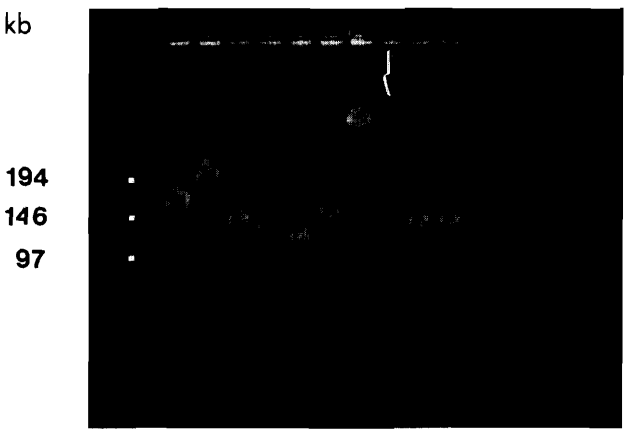
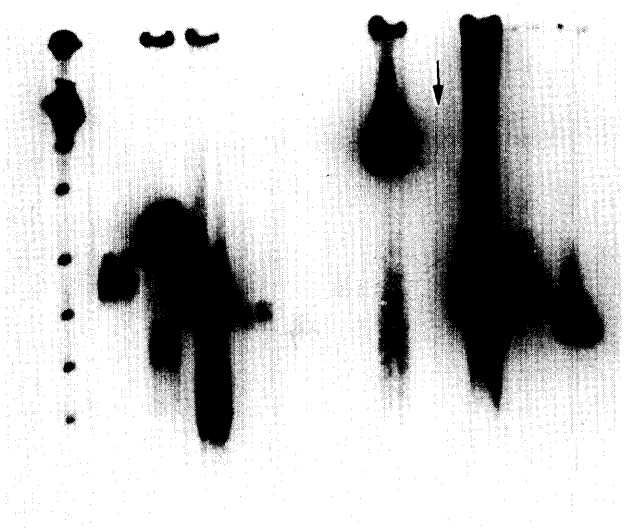


Fig. 2. Southern hybridization of the gel shown in Fig. 1 with radio-labeled *G. hirsutum* total genomic DNA. Signal for BAC 1928 (arrow) was not observed, indicating no or very little repetitive DNA content.



and cytogenetic landmarks. An essential step in development of such an integrated map is the accumulation of sufficient numbers of cytogenetic markers across the genome. Whereas many hundreds of loci have been physically mapped in humans by ISH of cosmids (Lichter et al. 1990; Hori et al. 1992; Takahashi et al. 1992) and yeast artificial chromosomes (Wada et al. 1990; Drieson et al. 1991; Moir et al. 1994), few gene loci have been physically mapped in higher plants, even when considered collectively.

The sparsity of physically mapped genes in plants is due in part to the limited success with which ISH has been used to detect single-copy plant DNA sequences. Although detection of single-copy sequences has been reported in a number of cases, detection has not been consistent or reliable (Jiang and Gill 1994). The use of large genomic DNA clones as ISH probes would forego problems associated with the sensitivity of plant ISH and presumably allow the physical mapping of many plant genomes. Of the two large DNA cloning systems generally available for plants, yeast artificial chromosomes (YACs) (Burke et al. 1987) and bacterial artificial chromosomes (BACs) (Shizuya et al. 1992), the latter offers a number of significant advantages from a molecular cytogenetic viewpoint. First, BAC DNA in the 50–350 kilobase (kb) size range can easily be isolated relatively free from host genomic DNA by standard plasmid minipreparations. Also, BAC libraries have a much lower percentage of chimeric clones than YAC libraries (Shizuya et al. 1992; Woo et al. 1994). And finally, the BAC cloning system is relatively flexible and efficient, allowing specific BAC fragments to be rapidly subcloned into smaller BACs for closer examination (Shizuya et al. 1992).

The development of molecular cytogenetic markers is of particular interest for plants that lack reliable methods of karyotyping. In contrast to most animals, plants tend to have metacentric and submetacentric chromosomes, but few acrocentric chromosomes, rendering them more difficult to karyotype. Moreover, many economically important plants are polyploid and have chromosomes that are very small and seemingly less than fully amenable to common banding procedures. Many of these limitations are exemplified by upland cotton (*Gossypium hirsutum* L.), a disomic tetraploid (AADD, $2n = 4x = 52$), in which the chromosomes are small, closely graded in size, and similar in near-metacentric morphology (Price et al. 1990). To date, the only reliable method for identifying all cotton chromosomes remains the meiotic analysis of cytogenetic stocks, namely translocation heterozygotes, monosomics, and monotelodisomics.

Use of the aforementioned cotton cytogenetic stocks for the unambiguous assignment of ISH signals to specific chromosome arm segments has previously been described (Crane et al. 1993). Preparation of meiotic metaphase I slides is time consuming, however, and many of these slides yield inferior ISH results compared with somatic metaphases from the protoplasted cells of root tips (our unpublished results). The identification of BAC clones specific for all chromosomes or all homoeologous pairs of cotton chromosomes would allow the use of mitotic metaphase preparations for mapping ISH signals, presumably increasing the rate and the sensitivity with which specific sequences could be physically mapped in cotton with ISH.

At this time, we report an approach that allows the reliable in situ hybridization and fluorescent detection of single-copy sequences on somatic plant chromosomes. Specifically, we demonstrate that a moderately large (130 kb) BAC containing mostly unique sequence *G. hirsutum* DNA can be used as a probe for the generation of strong single-copy FISH (fluorescence in situ hybridization) signals on the chromosomes of A-genome and D-genome cotton diploids, without the use of image processing. The technique is perceived as suitable for application across plant species.

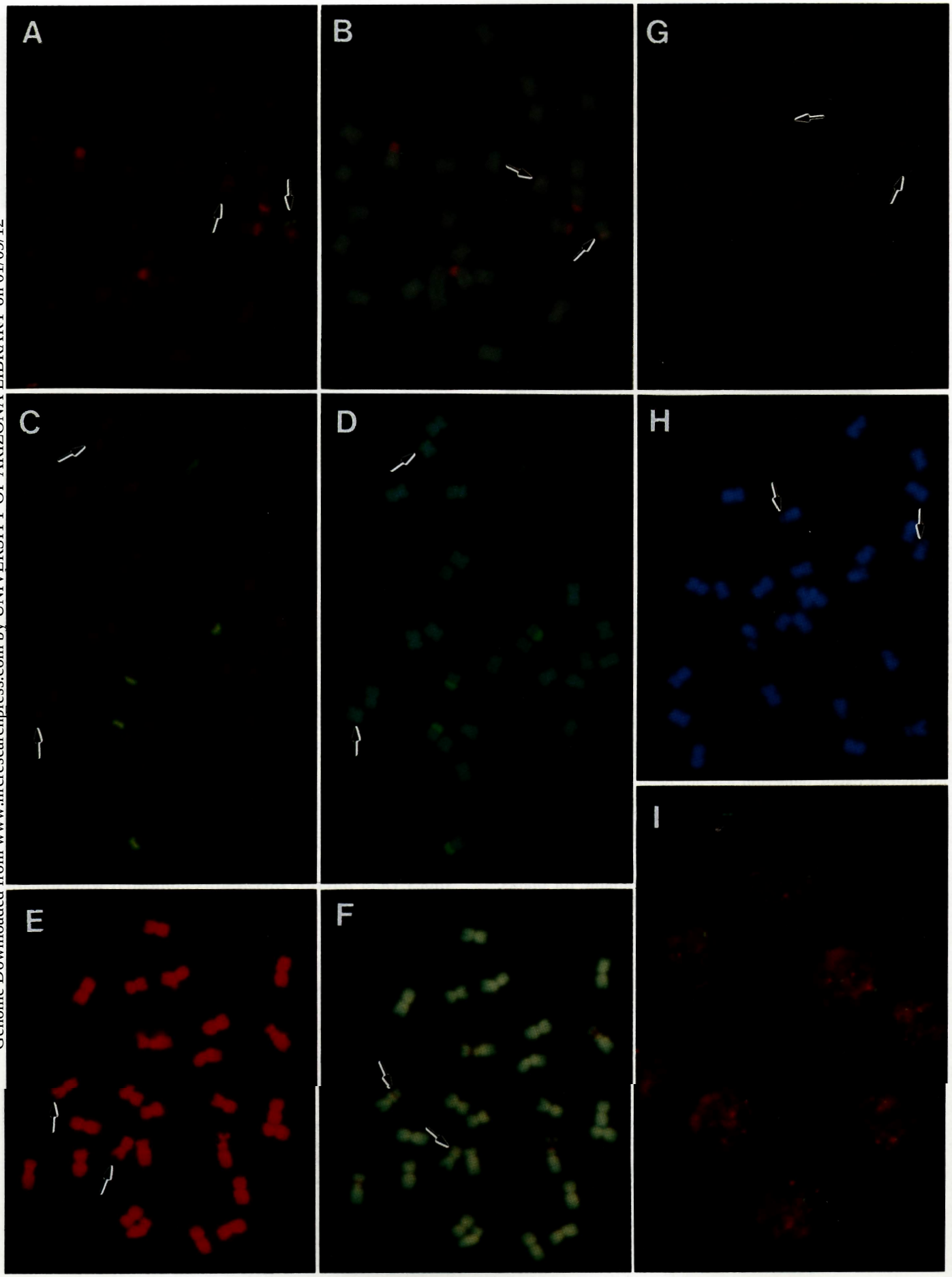


Fig. 3. Fluorescence images of ISH signals on chromosomes and nuclei from *Gossypium* diploids. (A and B) Paired images of *G. raimondii* chromosomes showing BAC 1928 FISH and NOR loci, respectively: (A) BAC 1928 FISH signals (arrows); (B) fluorescence of chromatin stained with DAPI and PI (30 min staining) showing PI stained minor NORs (arrows) syntenic to BAC signal (opposite arm) and two larger terminal or slightly subterminal NOR loci. (C and D) Paired images of *G. raimondii* chromosomes showing positions of NOR loci: (C) 18S–28S rDNA FISH signals showing a single minor NOR locus (arrows) and two major NOR loci; (D) fluorescence of DAPI-stained DNA (blue) and FITC-detected NORs (green) showing location of minor NOR locus (arrows) in the short arm. (E and F) Paired images of *G. herbaceum* chromosomes showing BAC 1928 FISH and NOR loci, respectively: (E) BAC 1928 FISH signals (arrows) on *G. herbaceum* chromosomes; (F) DAPI and PI (30 min staining) fluorescence of E showing a major NOR locus (arrows) syntenic to each BAC ISH site on a satellited chromosome; (G and H) Paired images of *G. herbaceum* chromosomes following reduction of PI staining (15 min staining) showing BAC 1928 FISH and NOR loci, respectively: (G) BAC 1928 FISH signals; (H) DAPI fluorescence showing satellited major NOR (arrows) syntenic to BAC ISH signals. Propidium iodide fluorescence is not visible owing to staining reduction. (I) BAC 1928 FISH signals on *G. raimondii* interphase nuclei.

Materials and methods

BAC cloning and screening

Gossypium hirsutum BACs were constructed and screened as described by Woo et al. (1994), with the exception that megabase DNA was isolated from nuclei preparations. Briefly, the screening procedure was as follows: 80 BACs were analyzed on CHEF (contour-clamped homogeneous electric field) gels; 11 of the 80 were selected based on large insert size; selected clones were run together on a CHEF gel, then stained with ethidium bromide, photographed, transferred onto a filter (Hybond-N^+, Amersham), and Southern hybridized separately with chloroplast DNA and cotton genomic DNA. BAC 1928 was selected for ISH on the basis of its screening negative for both chloroplast and repetitive sequence DNA.

Plant material and pretreatment

Gossypium raimondii and *G. herbaceum* root tips were treated with 2.5 mM 8-hydroxyquinoline (aq.) at RT for 3 h, then fixed in ethanol – acetic acid (3:1) fixative overnight at room temperature. *Gossypium herbaceum* and *G. raimondii* were chosen for demonstration of BAC FISH because of their close relationship to *G. hirsutum* (see Discussion) and their cytogenetic simplicity.

Metaphase preparation

Fixed root tips were rinsed in water for 10 min, and the meristematic regions of the root tips were then excised and placed in 15 μL of cell-wall lytic enzymes (5% cellulase, 2.5% pectolyase in 0.1 M citrate buffer) in 0.5 mL microcentrifuge tubes (5 meristems per tube). After a 40 min incubation at 37°C, meristems were individually rinsed with distilled water, placed on a clean glass slide with a drop of ethanol – acetic acid (3:1) fixative, teased apart with a fine-tipped pair of tweezers, and allowed to air-dry at RT for at least 2 days prior to use.

Probe DNA isolation and probe labeling

BAC and pGMR3 (a 4.5 kb *Eco*RI fragment of the 18S–28S ribosomal repeat of *Glycine max* in pBR325, kindly provided by Elizabeth Zimmer) DNA were isolated by alkaline lysis plasmid maxipreps as described by Silhary et al. (1984). Whole plasmid DNA was labeled with biotin 14-dATP (BRL) using the Gibco BRL BioNick Labeling System. The probes produced had an average fragment [illegible]pairs (bp).

C_0t-1 DNA preparation

The procedure of Nisson et al. (1991) was used to isolate C_0t-1 DNA from *G. hirsutum* genomic DNA. Briefly, DNA was sheared to a size similar to the probe, denatured, allowed to reanneal to a C_0t of 1, treated with S1 nuclease, and extracted with phenol–chloroform, followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

In situ hybridization

Slides were immersed in 30 μg/mL RNase in 2× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) for 45 min at 37°C and then denatured at 70°C in 70% formamide in 2× SSC for 2.5 min followed by dehydration in 70, 85, 95, and 100% ethanol for 2 min each at −20°C. Approximately 200 μL of probe mix (8 slides per experiment) was denatured at 80°C for 5 min, chilled on ice for 1 min, allowed to reanneal with blocking DNA for 5–30 min at 37°C, and then applied to the slide in a 25 μL volume and covered with a 20 × 40 mm coverslip, which was sealed with rubber cement. For BAC 1928, each 25 μL of hybridization mixture contained 50% deionized formamide, 10% dextran sulfate, 8 μg *Escherichia coli* DNA, 750 ng *G. hirsutum* C_0t-1 DNA, and 15 ng labeled BAC DNA in 2× SSC. For experiments without blocking, the C_0t-1 DNA was replaced with 1 μg *E. coli* DNA and the probe was not allowed to reanneal. For the 18S–28S probe, 90 ng of probe was used along with 10 μg *E. coli* carrier DNA, no C_0t-1 DNA, and the probe was not allowed to reanneal prior to application.

Following overnight incubation at 37°C, coverslips were floated off in 2× SSC and slides were rinsed at 40°C in: 2× SSC for 5 min, 2× SSC for 5 min, 2× SSC – 50% formamide for 10 min, 2× SSC for 5 min, 2× SSC for 5 min, and 4× SSC plus 0.02% Tween 20 for 5 min. Slides were blocked 5 min at RT with 5% (w/v) BSA in 4× SSC plus 0.2% Tween 20. Excess block was then blotted from the side of the slide and signal was detected with 60 μL of 5 μg/mL FITC-avidin DCS (Vector) – 4× SSC plus 0.2% Tween 20 for 30 min at 37°C. Slides were washed 3 times in 4× SSC plus 0.2% Tween 20 for 6 min at 37°C, blocked 5 min at RT with 5% normal goat serum in 4× SSC plus 0.2% Tween 20, blotted to remove excess block and amplified with 60 μL of 5 μg/mL biotinylated antiavidin D (Vector) – 4× SSC plus 0.2% Tween 20 for 30 min at 37°C followed by 3 washes in 4× SSC plus 0.2% Tween 20 at 37°C. Then, 60 μl of 5 μg/mL FITC-avidin – 4× SSC plus 0.2% Tween 20 was applied for 30 min at 37°C.

Following 3 washes in 4× SSC plus 0.2% Tween 20 (5 min each at 37°C) slides were stained in 2 μg/mL DAPI in McIlvaine's buffer (9 mM citric acid, 80 mM $Na_2HPO_4 \cdot H_2O$, 2.5 mM $MgCl_2$, pH 7.0) for 20 min at RT, destained 20 sec in 2× SSC, routinely stained with propidium iodide (PI; 20 μg/mL in 2× SSC) 30 min at RT, and destained 20 sec in 2× SSC. Finally, antifade was applied under a 22 × 40 mm coverslip that was sealed with clear enamel nail polish. Alternatively, we reduced the PI staining to 10–20 min to enhance the ease of FITC signal detection.

Metaphase observation and photography

Images were photographed directly on Fuji HG ASA 400 professional film with an Olympus Vanox epifluorescence microscope, using standard Olympus filter sets for ultraviolet (DAPI), violet (DAPI and PI), and blue (PI and FITC) excitation.

Results and discussion

Approximately 3000 *G. hirsutum* BAC clones have been established to date (Zwick et al. 1995), 11 of which were selected for this study on the basis of large insert size as determined by pulsed-field gel electrophoresis (Fig. 1). The average insert size of selected clones was approximately 130 kb. To determine the amount of repetitive DNA contained within the 11 BACs, either chloroplast or genomic, a single gel showing all 11 BACs (Fig. 1) was Southern blotted and hybridized sequentially with chloroplast DNA and *G. hirsutum* total genomic DNA. The results for the chloroplast DNA were negative for all clones (data not shown). Southern hybridization with *G. hirsutum* total genomic DNA (Fig. 2) indicated that 7 of the 11 clones contained highly repetitive DNA, 2 contained moderately repetitive DNA, 1 contained largely unique sequence DNA, and 1 clone of 130 kb, designated BAC 1928, contained almost all unique or low-copy DNA sequences.

In situ suppression hybridization of BAC 1928 to *G. raimondii* chromosomes consistently yielded a strong pair of signals located at the end of the long arm of a midsized submetacentric pair of chromosomes (Figs. 3A and 3B). Four discrete signals, one per chromatid, were visible in over 90% of the 70 metaphases analyzed. PI staining, which fluoresces brightly at the nucleolar organizer regions (NORs) of cotton chromosomes in many meiotic and mitotic preparations (unpublished results), indicated that the BAC ISH site was syntenic to a minor NOR site located on the opposite arm (Fig. 3B). To confirm the distribution of the NOR sites in *G. raimondii* inferred by PI staining, in situ hybridization of an 18S–28S rDNA probe was used. The ISH signal distribution was consistent with PI staining results in that it revealed two major NORs as well as a minor NOR site located near the centromere on the short arm of a midsized submetacentric pair of chromosomes (Figs. 3C and 3D).

ISH of BAC 1928 to *G. herbaceum* chromosomes gave results similar to those obtained for *G. raimondii*, but the FISH signals were considerably smaller. The signals were located on the distal end of the long arm of a small, submetacentric pair of chromosomes (Figs. 3E–3H). BAC ISH signals occurred on all four chromatids in approximately 65% of the 40 metaphase spreads stained under the routine PI staining protocol. Chromosome morphology (Figs. 3F and 3H) and PI staining (Fig. 3F) demonstrated that the BAC ISH signals were located on one of three major NOR-bearing pairs of chromosomes. When PI staining was decreased, BAC 1928 signals became more pronounced on most metaphase chromosomes (Fig. 3G) and interphase nuclei and were detectable on all four chromatids in approximately 80% of the 30 metaphase cells analyzed.

Our repetitive DNA screening procedure indicated that BAC 1928 was largely, if not completely, devoid of repetitive sequences. To further examine this finding, BAC 1928 was hybridized without any C_0t-1 DNA blocking to both the A- and D-genome cotton diploids. Again, large signals occurred on one pair of *G. raimondii* chromosomes, but extremely small signals also occurred, interspersed throughout the chromosomes. Distinct BAC ISH signals were detected at high frequency in interphase cells (Fig. 3I) and metaphase cells of *G. raimondii*. One pair of moderately sized signals was seen in *G. herbaceum* and no repetitive signal was visible (data not shown).

Gossypium hirsutum is an AD allotetraploid ($2n = 4x = 52$) originating from an interspecific hybridization event(s) between two diploid *Gossypium* species most closely related to the A-genome species *G. herbaceum* and the D-genome species *G. raimondii* (Endrizzi et al. 1985). The A-genome and D-genome species are estimated to have begun divergence from a common ancestor between 6 and 11 million years ago and the hybridization giving rise to *G. hirsutum* is estimated to have occurred 1–2 million years ago (Wendel 1989; Wendel and Albert 1992). The 2C DNA contents of these A-genome, D-genome, and AD-genome species are 3.81, 2.19, and 5.68 pg, respectively (Edwards et al. 1974; Gomez et al. 1993). Kinetic DNA analysis of *G. herbaceum* and *G. raimondii* has shown that the difference in DNA content between the A and D genomes is due to repetitive DNA and that little difference exists between the two in single-copy DNA content (Geever et al. 1989). This information, combined with the differences in signal intensities between the A and D genomes, as well as the detection of repetitive signals only on *G. raimondii* chromosomes when using no C_0t-1 DNA blocking, suggests a D-genome origin for BAC 1928.

Owing to the fact that the whole BAC 1928 plasmid, including the plasmid vector, was nick translated and included in the probe mix, it is conceivable that the plasmid could contribute to some observable ISH signals. This is unlikely however, for two reasons. First, the BAC plasmid is of prokaryotic origin and presumably has little homology with any cotton sequences. Second, and confirming the previous assumption, is the fact that the BAC plasmid signal was negligible when Southern hybridized with *Gossypium* genomic DNA (Fig. 2).

In summary, we have demonstrated that bacterial artificial chromosome clones can be used as FISH probes for the development of new plant molecular cytogenetic markers. Additionally, it has been demonstrated that as a molecular cytogenetic marker, a BAC can yield a high signal-to-noise ratio without the aid of image processing, even when using a genomic clone only moderately large in size (130 kb). Based on the sizes and frequencies of BAC 1928 ISH

signals, as well as our unpublished results with other BACs, we anticipate FISH detection will be routine for BACs containing unique sequences of 30 kb and possibly smaller in size. These results, combined with the efficiency of the BAC cloning system, suggest that FISH of BAC clones will become an important addition to the effort to physically map many crop-species genomes and may help to revolutionize the field of plant cytogenetics.

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