GENOMIC ANALYSIS OF SORGHUM

BY FLUORESCENCE *IN SITU* HYBRIDIZATION

A Dissertation

by

JEONG-SOON KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Plant Breeding

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Approved as to style and content by:

David M. Stelly	H. James Price
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ABSTRACT

Genomic Analysis of Sorghum by Fluorescence *In Situ* Hybridization. (August 2003)

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The reliability of genome analysis and proficiency of genetic manipulation *in vivo* and *in vitro* are increased by assignment of linkage groups to specific chromosomes, placement of centromeres, orientation with respect to telomeres, and linear alignment with respect to chromosomal features and dimensions. I undertook five studies aimed at integrating sorghum genomics and cytogenetics at several levels. The results help establish an entirely new "cyto-genomics" resource, impacts of which are likely to be broad. In the first study, I developed a FISH-based karyotyping system for *Sorghum bicolor* Moench. I used integrated structural genomic resources, including linkage maps and large-insert clonal libraries of sorghum genomic DNA to develop a 17-locus probe cocktail for simultaneous fluorescent *in situ* hybridization (FISH). This probe enabled facile identification of all chromosome pairs in mitotic chromosome spreads. Perhaps just as important, I established time-efficient means to select sorghum BAC clones for multi-probe FISH. Thus, an integrated cyto-genomics system for sorghum can be constructed without need of chromosome flow sorting or microdissection, both of which are difficult and costly. In the second study, hybridization of DNA clones from 37

different genomic regions enabled the assignment of linkage groups and orientation of linkage maps to chromosomes. Comparisons between genetic and physical distances throughout the genome enabled a new nomenclature for linkage group designation in sorghum. The results provide an integrated nomenclature system of *Sorghum bicolor* chromosomes and linkage groups. In the third study, I created high-resolution maps by FISH to pachytene bivalents for two linkage groups (B and H), and defined relationships between pericentromeric heterochromatin, centromeres, mapped markers and recombination rates. These relationships will help guide the development and use of sorghum genomics. In the fifth study, I used FISH in two ongoing gene-targeted efforts. For the maturity gene ma_5 and fertility restoration gene *rfl*, I estimated physical lengths between currently available flanking molecular markers. This enables estimation of recombination densities in these regions and assessment of the applicability of map-based and -assisted cloning.

To my husband, Min Ahn, and my babies, Yerim and Moojin.

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I would like to express my appreciation and love to my parents and lovely husband, Min Ahn, for their endless love, support and encouragement. I can't imagine this completion without them. Finally, I would like to thank my God for being always with me and bringing me strength and patience.

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CHAPTER I

INTRODUCTION

Genomic maps of specific genes, DNA markers and other genomic sequences are among the most important resources for genetic improvement of domesticated animals and plants. Most eukaryotic genome maps can be categorized as recombinational (*e.g.*, linkage), physical (*e.g.*, contigs to sequences), or cytological (chromosomal to fibrous DNA). Two or more of these maps can be related to each other to form an integrated map. The integration of maps typically enhances reliability and robustness of individual maps, and extends their utility. Upon integration, linkage maps become more useful at the molecular level, and physical maps become a valuable resource for linkage marker and map development. All three types of maps predict order, but at varied levels of resolution and perspective. Thus, each of them provides a different view of syntenic relationships. Whereas linkage maps have direct predictive value for in vivo recombination, physical and cytological maps provide molecular, chromosomal, whole-genome and nuclear perspectives that cannot be deduced from linkage maps alone.

Linkage maps directly enhance our ability to dissect and characterize the genetic control of complex traits with respect to the number of loci, alleles, modes of action, genetic interactions, and transmission. However, many factors commonly limit utility of linkage maps, such as insufficient marker types, density and portability across genotypes and genera, incomplete and variable coverage, highly variable recombination density,

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This dissertation follows the style and format of Genome.

limited numbers of common markers among maps, mapping errors, and a lack of knowledge concerning the relationship between linkage map loci and chromosomal position, *e.g.*, coverage and orientation. In most cases, linkage maps are low in resolution relative to physical maps, and vary widely from being much higher to much lower in resolution than cytological maps. It seems that recombination is relatively frequent in most gene-rich regions, but this varies by region and homology; moreover, the practical significance in terms of transmission is greatly dependent on cytogenetic milieu. Thus, the practical utility of linkage maps for wide-crosses and interspecific germplasm introgression can be unreliable and is typically unpredictable.

Physical maps describe overlapping clonal relationships and enable effective usage of clonal libraries for sequencing, molecular analysis, manipulation and comparison. However, there is growing evidence that molecular maps relate poorly to recombination, other than for order of loci. If not related to other types of maps, physical maps of eukaryotic genomes are a collection of many variably sized high-resolution maps, but collectively highly fragmented. In autonomous form, their disjointed composition severely limits their utility for any studies that require a perspective that requires anything more than homology relationships and immediately neighboring clones, *e.g.,* genetic, recombinational, syntenic, chromosomal, subgenomic, genomic, intergenomic, or spatial relationships. Alone, physical maps tell us relatively little about the significance of genomic organization or chromatin milieu. Many of these limitations are removed or circumvented by map integration, and greatly enhance the value of sequence data and related information for scholarly inference and practical application.

Molecular cytogenetic maps provide a perspective that is uniquely genome-wide and easily extended to related genomes. The maps enable direct and quick detection of gross structural differences of related genomes at mitosis and/or meiosis, without requiring hybridization or creation of a segregating population for linkage mapping. However, if hybridized, molecular cytogenetic analysis of meiotic or post-meiotic F1 stages allows rapid assessment of recombinational relationships, unfettered by interference of gametophytic or embryonic lethals. Although the molecular cytogenetic resolution varies widely according target, it typically exceeds resolution of linkage maps in large low-recombination regions. However, it is far lower than physical map resolution. Physical genome maps derived from clonal resources are highly fragmented, because many regions are not readily or stably cloned. In contrast, all regions, clonable or not, are visible cytologically. The comprehensive perspective offered by FISH is thus unique and valuable. The targets of FISH can be chromosomal, nuclear or fibrous DNA, and thus tailored to the level of resolution desired. The objectives described herein require only chromosomal FISH, to which subsequent discussion is therefore relegated.

Development of molecular marker technology revolutionized genetic linkage mapping. In contrast to genetic mapping, much less effort has been devoted to physical mapping during the last decade partly due to the lack of techniques that are affordable to many labs and that can be applied to different species. Thus, the majority of the genetic linkage maps developed in plant species have yet to be integrated with any type of physical map. Currently, three popular methods for physical mapping are used in plant species; DNA contigs using large insert DNA clones, mapping DNA markers to specific

chromosomal segments using cytogenetic stocks and fluorescence *in situ* hybridization (FISH). The technique of fluorescence *in situ* hybridization (FISH) to chromosomes provides the most direct, rapid way to establish a physical map. Since *in situ* hybridization was developed (Gall and Pardue 1969; John et al. 1969), it has expanded into many fields of genetics and cell biology, *e.g.*, aneuploid identification (Ji et al. 1997, 1999a), polyploid genome evolution (Hanson et al. 1998), meiotic recombination (Ji et al. 1999b), recombination analysis (Reyes-Valdes et al. 1996), karyotyping (Chen et al. 2000; Dong et al. 2000), genomic introgression (Jacobsen et al. 1995), physical mapping (de Jong et al. 1999; Sadder et al. 2000). Application of *in situ* hybridization has been hindered by the low sensitivity of the technique in plant chromosomal preparations. It has been technically difficult to detect small probes containing only a few kilobases of DNA using *in situ* hybridization. Thus, the majority of the markers used in genetic linkage mapping are not suitable for direct use as probes for *in situ* hybridization analysis. This technical difficulty can be partially overcome by using larger pieces of DNA as FISH probes, such large-insert DNA clones. However, repetitive DNA sequences typically found within large-insert DNA clones can lead to a great deal of location-nonspecific FISH signal that is distributed widely across the genome. Thus, the strategy conjures up a new set of technical difficulties for FISH analysis. Nevertheless, the majority of randomly selected or RFLP marker-selected BAC clones can be used for FISH mapping in at least some plant species, *e.g.*, Arabidopsis, rice and potato and sorghum (Jiang et al. 1995; Fransz et al. 1998; Zwick et al. 1998; Dong et al. 2000).

The first materials to be used for FISH experiments were mitotic metaphase chromosomes. However, resolution remained limited, generally in the order of 2-5 Mbp (Trask 1991; Jiang et al. 1996). Meiotic pachytene chromosomes, which are often more than 10 times longer than somatic metaphase chromosomes, have recently been used as a target for FISH mapping (Xu and Earle 1996; Fransz et al. 1998, 2000; Peterson et al. 1999; Chen et al. 2000). The high resolution of the pachytene FISH method, together with the recently developed fiber-FISH techniques (Fransz et al. 1996; Jackson et al. 1998, 2000), adds new tools to the arsenal for fine physical mapping.

The development of reliable and simple techniques for chromosome identification is critical for genome analysis and cytogenetics. Identification of individual chromosomes and segments using banding techniques has revolutionized cytogenetics research for humans, many other mammals and several plant species. Unfortunately, chromosome identification is a major challenge in many species with small chromosomes, including numerous plant taxa. With fewer bands, it is far more difficult to distinguish chromosomes and detect rearrangements. With the advent of non-isotopic *in situ* hybridization techniques in plants, a single repetitive DNA probe could be used for molecular cytogenetic karyotyping and chromosome identification. However, such repetitive DNA probes have been developed for few plant species (Armstrong et al. 1998; Dhar et al. 2002). An alternative and more flexible approach is to identify chromosomes and sub-regions by producing identifiable FISH signals from tagged large-insert DNA clones.

Sorghum (*Sorghum bicolor* (L.) Moench, $2n = 20$) is a domesticated grass grown in many tropical and subtropical regions. Its importance is accentuated in many agriculturally recalcitrant semi-arid regions, *e.g.*, in Africa (Quinby 1974). This grain crop is the fifth most important cereal grown worldwide, due in large part to its unusual tolerance to adverse environments (Doggett 1988). For this reason and others, the genetic and physiological basis of sorghums environmental stress tolerance has been the subject of numerous investigations (Blum et al. 1990; Crasta et al. 1999; Salih et al. 1999). More recently, plant scientists interested in genomics have been attracted to sorghum because its genome is small (750 Mbp) relative to most grasses such as maize (*Zea mays*, 2,400 Mbp) and wheat (*Triticum aestivum*, 16,000 Mbp) - with the exception of rice (*Oryza sativa*, 430 Mbp) (Arumuganathan and Earle 1991). Over the past several years, high-resolution genetic maps of sorghum have been generated (Chittenden et al. 1994; Hulbert et al. 1990; Peng et al. 1999; Pereira et al. 1994; Xu et al. 1994) and sorghum BAC libraries constructed (Woo et al. 1994; Tao and Zhang 1998). The entire set of 26,000 BAC clones was fingerprinted and contigs based on the fingerprint data were assembled (Klein et al. 2000). In addition, a high-throughput PCR-based screening method was developed which combines six-fold BAC DNA pooling and amplified fragment length polymorphism (AFLP) technology (Klein et al. 2000). This methodology allowed us to identify BAC clones that contain genetic markers and, in most cases, are associated with a specific contig. Thus, FISH with these clones could generate an integrated resource including cytological, physical (contig) and linkage maps.

This approach provides a low cost, efficient way to build high-quality integrated genetic and physical genome maps.

Cytogenetic characterization of the ten sorghum chromosomes would complement and enhance the genetic and physical information being compiled for sorghum. Cytological determination of chromosomal infrastructure such as centromeres, NORs, and heterochromatin, and determination of the variation in recombination frequency across chromosomes would facilitate the use of these maps for comparative genomics and map-based gene isolation. Yu et al. (1991) identified every chromosome of Combine Kafir 60, a sorghum cultivar, using a Giemsa C-banding technique in conjunction with chromosome length and arm ratio measurements. Such techniques for karyotypic analysis, however, require rigorous testing to verify the fidelity of identification. The utility of BACs as molecular cytogenetic probes in plants became evident through early studies using marker-selected BACs for FISH (Hanson et al. 1995; Jiang et al. 1995). Utilizing marker-selected BACs from euchromatin in the distal regions of sorghum chromosomes, Gomez et al. (1997) used FISH to show that a 205 kb sorghum BAC containing a sequence complementary to the maize *sh2* cDNA produced strong signals at the distal end of one arm of a pair of mid-sized metacentric sorghum chromosomes. Zwick et al. (1998) used six liguleless-associated rice RFLP markers to select related sorghum BACs, which in turn were used to physically map a homologous region in the sorghum genome. FISH of the marker-selected BACs yielded signals on the distal region of a single chromosome arm. With one exception, their relative positions indicated that the order of the loci in sorghum was the same as in the rice

genetic map. The BACs were end-cloned for RFLP mapping, and the relative linkage order in linkage group I (Xu et al. 1994) was fully concordant with the observed order of FISH markers.

One of the applications for which integrated cytological maps can be highly advantageous is map-based or map-aided cloning. The sorghum industry is completely reliant on hybrid seed produced using cytoplasmic male sterility and nuclear alleles for fertility restoration. The identification and development of male-sterile and fertility restorer lines is essential for hybrid seed production in sorghum. The availability of molecular markers will facilitate the selection of pollen fertility restoration in sorghum inbred-line development and provide the foundation for map-facilitated gene isolation. The mapping and tagging of the *rf1* locus in sorghum by amplified fragment length polymorphism (AFLP) and microsatellite simple sequence repeat (SSR) genetic markers have been reported. A regional linkage map around the *rf1* locus was established, wherein *rf1* mapped to a position 2.4 cM from AFLP marker Xtxa 2582. It was determined that the *rf1* locus maps to linkage group H of the high-density genetic map of sorghum. FISH using BAC clones containing the genetic markers flanking the *rf1* locus will reveal the chromosomal order of the respective linkage markers and allowed comparison to their linkage map order. It will be possible to use the physical distances between FISH sites to estimate the molecular size of the respective linkage map segment. If small, *e.g.*, <1 Mb, the finding will strongly aid the strategic use of BAC contigs to span the *rf1* locus and provide a starting point for identifying the responsible gene(s).

The basic structure of eukaryotic chromosomes comprises two types of chromatin; euchromatin and heterochromatin. In sorghum, the majority of heterochromatin in each chromosome occurs as a "block" of pericentromeric heterochromatin. At certain stages these pericentromeric regions correspond to intensely stained DAPI-positive regions. However the degree to which they are differentially stainable is minimal at metaphase. This is when relative sizes of euchromatin and heterochromatin are most comparable, because, both are maximally contracted. To understand the organization of the euchromatic and heterochromatic components of an eukaryotic chromosome, it is necessary to relate the morphological features of chromosomes with genetic and the molecular sequence data. The resulting integrated map will provide tools and information that enable correlations to be established between DNA sequences, structure, and function of the chromosome. It will aid efforts to determine the mechanism behind chromatin condensation, recombination suppression and gene silencing of heterochromatin. It will also allow analysis of the overall architecture of the genome, including the size and distribution of the gene islands, the gene densities within these, and the range of gene structures.

CHAPTER II

INTEGRATED KARYOTYPING OF SORGHUM BY *IN SITU* HYBRIDIZATION

OF LANDED BACs*

Introduction

Reliable cytological techniques for chromosome identification are necessary for efficient genome research and germplasm utilization. The discoveries of mitogens, hypotonic bursting and chromosome banding that led to facile methods of mitotic cell recovery, chromosome spreading and segmental identification collectively revolutionized the cytogenetics of humans and many other animals (Hsu 1979). FISH-based physical mapping of repeated sequences, genomic clones, and cDNAs has been an important feature of human genomics. Chromosomal and subchromosomal localization of breakpoints and *in situ* hybridized probes relied on a backdrop of routinely produced karyotypes comprised of 400+ bands (Lichter et al. 1990; Gingrich et al. 1993; Moir et al. 1994; Muleris et al. 1994). More recently, close to 9,000 BACs of the Human Genome Project have been analyzed by FISH to confirm their integrity and point of origin, to crosscheck critical framework maps, to reveal duplications and paralogy, and to provide

an integrated resource for genomics and gene identification, *e.g.*, positional candidate gene identification for traits with known cytogenetic aberrations (Cheung et al. 2001). These operations underscore the feasibility of using large-insert libraries and integrated genomics resources for development of molecular cytogenetic resources.

Chromosome banding methods have unfortunately been far less effective in most plants than in mammals and have severely limited the utility of conventional karyotypic analysis in development of plant genomics. Efficient karyotypic methods to screen for segmental chromosomal variation are still lacking in all but a few species with very large chromosomes (*e.g.*, wheat $2n = 42$, ca. 16,000 Mbp/1C). Innovative approaches offer useful alternatives in some species, *e.g.*, maize-oat addition line radiation hybrids (Riera-Lizarazu et al. 2000) and wheat gametocidal system-induced segmental deletion lines (Endo and Gill 1996), but solutions offering general applicability are desirable. In most plant species, a detailed cytogenetic framework that would otherwise expedite the development and integration of genomics resources is still lacking. The need for such capabilities in plant genomics is acute, due to the prevalence of polyploidy among plants. For example, the rate among angiosperms is in the range of 40 - 70% (Stebbins 1950; Masterson 1994). Recent evidence of polyploid features in angiosperms heretofore widely regarded as diploid, *e.g.*, maize (Moore et al. 1995) and arabidopsis (Blanc et al. 2000; Arabidopsis Genome Initiative 2000), suggests that rates of polyploidy among angiosperms are higher than previously suspected. The dosage and organizational changes resulting from polyploidy, segmental duplications, deletions, and rearrangements are of significant concern due to their effects on genome mapping, clonal

library coverage, screening efficacy, contig assembly, and the uniqueness of genes and gene products. The importance of karyotypic systems and integrative genome mapping is thus especially important in plant genomics and genome manipulation.

Sorghum (*Sorghum bicolor* (L.) Moench, $2n = 20$) is a domesticated grass grown in many tropical and subtropical regions. Its importance is accentuated in many agriculturally recalcitrant semi-arid regions, *e.g.*, in Africa. Yu et al. (1991) identified every chromosome of Combine Kafir 60, a sorghum cultivar, using a Giemsa C-banding technique in conjunction with chromosome length and arm ratio measurements. While the work of Yu et al. (1991) was a significant step forward, contemporary genomics research and breeding efforts require development of integrated genome analysis tools with resolution greater than C-banding can provide. Moreover, cytogenetic variability across germplasm might compromise the applicability of C-band-based karyotypes. In theory, FISH signals could serve as excellent cytological markers for chromosome or segment identification based on locus-specific FISH signals. Thus, we endeavor to develop a FISH-based karyotyping system for sorghum and other gramineous species. Herein, we report results of one of the first steps -- tagging each of the 10 sorghum chromosome pairs in a karyotypically distinct manner.

There are essentially two strategies for FISH-based karyotyping. The simpler one is based on patterns from one or more multi-locus repeated sequences that yield FISH signal at a limited number of discrete loci, as exemplified by multicolor FISH of two or three tandemized repetitive sequences of rDNAs and satellite in spruce (Brown and Carlson 1997; Brown et al. 1998; http://dendrome.ucdavis.edu/Image/kary2.html).

Randomly arranged repeated sequences such as rDNA genes that are at one or a few loci in the genome can serve as robust FISH-based karyotypic markers. However, the rarity of such loci limits their utility -- in sorghum there is just one large 18S-5.8S-26S rRNA gene cluster (Sang and Liang 2000) and just one moderately sized 5S rRNA gene cluster (unpublished results). A second strategy for FISH-based karyotyping could be based on collective use of multiple low-copy sequences, each of which hybridizes to just one location per genome, or perhaps a few, due to duplications and/or polyploidy. Low-copy clones are potentially a much more plentiful source of probes, especially given the rapid development of large-insert libraries and related genomic infrastructure in many taxonomic groups. To achieve robust FISH signals from low-copy sites, mid-sized (cosmid) and large-insert DNA clones, *e.g.*, BACs (Lichter et al. 1990; Hori et al. 1992; Takahashi et al. 1992; Hanson et al. 1995; Fillon et al. 1998; Song et al. 2000; Dong et al. 2000) can be used as cytological markers to tag individual chromosomes. Many large-insert genomic clones that contain low-copy sequences also contain repetitive sequences that hamper or preclude detection of the low-copy sequences by FISH. When homologous repetitive sequences are abundant in the target genome and the BAC probe, BAC-FISH often results in FISH signal that is widely distributed across the genome. Such signal creates "noise" that essentially camouflages signal occurring at the BAC-homologous sequence locus. While "blocking" with unlabeled Cot1 or other repetitive fractions can alleviate moderate noise, it is insufficient in some instances. Moreover, the process is protected by patent (US Patent No. 5,447,841).

FISH of most plant BACs commonly results in excessive "noise" from multi-site

hybridization of repeated sequences. For some BACs, a remedy for FISH "noise" is competitive *in situ* suppression (CISS) with blocking DNA, usually unlabeled Cot1 fraction genomic DNA. For other BACs, however, excess blocking DNA cannot adequately preclude hybridization by labeled probe that contains repeated sequences, so background hybridization patterns remain significant. Thus, when discrete FISH loci are sought, it is helpful to select clones containing relatively large amounts of unique sequences and relatively small amounts of dispersed repetitive sequences. Similar considerations guided the production of region-specific FISH probes from flow-sorted chromosomes, micro-dissected chromatin and other large-insert clones, such as YACs.

In sorghum, regions adjacent to centromeres are largely heterochromatic and regions near the ends of chromosomes are euchromatic. In previous work on sorghum, most BACs yielding discrete FISH signals originated from the ends of chromosome arms, *i.e.*, from euchromatic regions (Zwick et al. 1998; Gomez 1997). As one of the steps in development of a facile molecular cytogenetic system for sorghum, we devised and tested strategies that would expedite development of BAC probes with the ability to yield discrete high-quality signal after FISH, *i.e.*, "FISHable" BACs. To identify FISHable BACs, we selected BACs containing markers located near the ends of each of the individual linkage group. Here we report the selection of BAC probes that can be used for FISH. The probes provide an excellent means for sorghum chromosome identification, for initial integration of structural and functional genomics resources with chromosomal features, and to initiate development of a robust molecular cytogenetic system for sorghum and other gramineous species.

Materials and Methods

Selection of BACs for FISH

The BACs used in this study came from two genomic BAC libraries that derived from sorghum cultivar BTx623, which collectively contain 26,016 clones with an average insert size of *ca.* 150 Kbp, *i.e.*, about 5X sorghum genome equivalents (Tao and Zhang 1998; Woo et al. 1994). An "sbb" (Sorghum bicolor BAC) number refers to the BACs from these libraries. The BACs from the Woo et al. (1994) library are numbered sbb1 to sbb13440, and the BACs from the Tao and Zhang (1998) library are numbered sbb13441 to sbb26016.

Molecular markers from a high-density genetic map of sorghum were used to screen a complex set of BAC pools (Klein et al. 2000; Menz 2002). To increase the likelihood that syntenic BAC FISH loci would be distinguishable from each other after hybridization to chromosomes, the markers from a common linkage group were selected only if separated by a substantial genetic distance. Additionally, only markers that mapped to regions of apparently high recombination near the ends of linkage groups were considered for use in this study. For BACs selected by AFLP or SSR markers, the primers for the appropriate AFLP or SSRs were used for screening the BAC pools by PCR. For BACs selected by RFLP markers, the clone for the RFLP was sequenced, and specific primers were designed for the RFLP clone. The RFLP-specific primers were then used to screen the BAC pools by PCR. In all cases, BACs initially identified by the results from BAC pool screening were individually re-screened using PCR primers for

the marker that identified that BAC. Most of the BACs, used as FISH probes, were identified by direct association with mapped markers. However, five of the BACs (sbb19188, sbb10186, sbb10491, sbb10718, and sbb12906), used as probes, were indirectly identified by mapped markers as members of fingerprinted BAC contigs that were identified by a mapped marker. BAC sbb3766 was used because it contains the phytochrome *PHYA* gene and has been fully sequenced (Genbank accession AF369906; D.T. Morishige, K.L. Childs, J.E. Mullet, unpublished data).

Metaphase preparation

Sorghum (*Sorghum bicolor* [L.] Moench.) plants (BTx623) were grown under glasshouse conditions. Excised root tips were treated with saturated aqueous α-monobromonaphthalene solution at RT for 1.5 hr, fixed in ethanol-acetic acid (3:1) fixative, and rinsed in water for 10 min. Excised meristematic tips were placed in enzyme solution (5% cellulase, 2.5% pectolyase in 0.1 M citrate buffer) at 37°C for 40 min, rinsed with distilled water, placed on a clean glass slide with a drop of ethanol-acetic acid (3:1) fixative, macerated using fine-pointed forceps, allowed to air dry at RT for two days, and stored in a −20°C freezer.

BAC DNA purification and probe labeling

BAC DNA was isolated by alkaline lysis, digested with *Eco*RI, and then further purified using Plant DNeasy spin columns (Qiagen, Valencia, CA) using a modified protocol (Childs et al. 2001). The purified BAC DNA was labeled with biotin 16-dUTP

or digoxigenin-11-dUTP by the BioNick Labeling system (Roche Molecular Biochemicals, Indianapolis, Indiana, USA).

In situ hybridization

In situ hybridization techniques were a modification of Jewell and Islam-Faridi (1994) as described by Hanson et al. (1996). Slides were immersed in 30 μg/ml RNase in 2 × SSC for 45 min at 37°C and then washed in 2 × SSC. The chromosomal DNA on the glass slide then was denatured at 70°C in 70% formamide in 2 × SSC for 1.5 min followed by dehydration in 70, 85, 95, and 100% ethanol for 2 min each at −20°C. The hybridization mixture (25 μl for one slide) contained 10 ng of labeled probe DNA, 50% formamide, 10% dextran sulfate, 2 × SSC. The mixture was denatured at 90°C for 10 min, chilled on ice, and added to the slide. Following overnight incubation at 37°C, slides were rinsed at 40°C in 2 × SSC, 50% formamide in 2 × SSC, 2 × SSC, 4 × SSC plus 0.2% Tween 20 for 5 min each. Slides were blocked 5 min at RT with 5% (w/v) BSA in 4 × SSC plus 0.2% Tween 20. Biotin-labeled probes were detected with 1% Cy3-conjugated streptoavidin and digoxygenin-labeled probes with 1% fluorescein isothiocyanate (FITC)-conjugated anti-digoxygenin antibody. Slides were washed three times in 4 × SSC plus 0.2% Tween 20 for 5 min at 37°C. DAPI in McIlvaine's buffer (9 mM citric acid, 80 mM $Na_2HPO_4^{.}H_2O$, 2.5 mM $MgCl_2$, pH 7.0) was used for counterstaining chromosomes. After rinsing, Vectashield antifade solution (Vector Laboratories, Inc.) was applied.

Microscopy

Images were viewed through an Olympus AX-70 epifluorescence microscope equipped with standard filter cubes. Prior to acquiring a digital imaging system, images were recorded photographically using Fuji HG ASA 400 professional film. Later, images from a Peltier-cooled 1.3 M pixel Sensys camera (Roper Scientific) were captured with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, California, USA.).

Results

Isolation of chromosome-specific BAC clones of sorghum

Marker-based BAC screening is generally assumed to be error-free, but errors could arise from infidelity of methods, erroneous data handling, or redundancies within genomes, *i.e*., repeated sequences, segmental duplications, or polyploidy. To minimize the possibility of such errors in this study, all BACs identified by screening the BAC DNA pools with mapped markers were determined to truly contain the mapped markers by rescreening the individual BAC DNAs (Fig. 1). In all cases tested, the expected markers were clearly present in the selected BACs.

BAC-FISH signal strength and distribution

Twenty-two sorghum BACs that were placed on the ten linkage groups of a high-density sorghum genetic map (Menz et al. 2002) were selected according to map position and tested by FISH for repetitive sequences. FISH of probes from individual BACs indicated that 19 of the 22 clones produced little or no background signal when hybridized to sorghum chromosomes *without* the aid of Cot1 DNA for blocking (Fig. 2). Only three clones resulted in moderate background signal when hybridized without blocking DNA (data not shown). One of the three BACs, sbb3766, has been fully sequenced and is known to contain five retrotransposon-like elements, along with eighteen genes (Genbank accession AF369906; D.T. Morishige, K.L. Childs, J.E. Mullet, unpublished data).

BAC-FISH signal synteny

To develop a pilot karyotyping system for the simultaneous identification of all sorghum chromosomes, we needed to develop distribution/color "signatures" of individual or pairs of syntenic BAC-FISH probes that would be diagnostic for each chromosome. To test the expected synteny of BACs selected with markers from a common linkage group, we used two-color detection of FISH for complementarily labeled pairs and sets of probes. Each pair of set of BACs associated with linked marker loci yielded FISH signals on a common chromosome pair (Figs. 3A-3D).

Fig. 1. Confirmation of BAC selection. BACs that were selected by screening each BAC pools with AFLP markers were individually checked to verify the presence marker in the respective BAC. The four sorghum BACs sbb6585, sbb9251, sbb17392 and sbb24713 were identified when the BAC pools were screened using primers that amplified AFLP markers *Xtxa224* (LG-A), *Xtxa516* (LG-D), *Xtxa391* (LG-B) and *Xtxa567* (LG-F), respectively. Each of these BACs was isolated, prepared as AFLP template and used in an AFLP reaction with the appropriate, fluorescently labeled AFLP primers (*Xtxa224*, E-CAA + M-CTG; *Xtxa516*, E-TGA + M-CCT; *Xtxa391*, E-TGA + M-CGA; *Xtxa567*, E-TGA + M-CGC). AFLP-template DNA from the mapping parents (IS3620C and BTx623) were also used in the same AFLP reactions. Fluorescently labeled products were run on a LI-COR DNA sequencer. The figure shows the gel images from these AFLP reactions. Marker names are given adjacent to the bands that are polymorphic between the mapping parents and that were specifically amplified from the individual BACs. Molecular weight markers are shown in lane M. All reactions were performed according to the method of Klein et al. (2000).
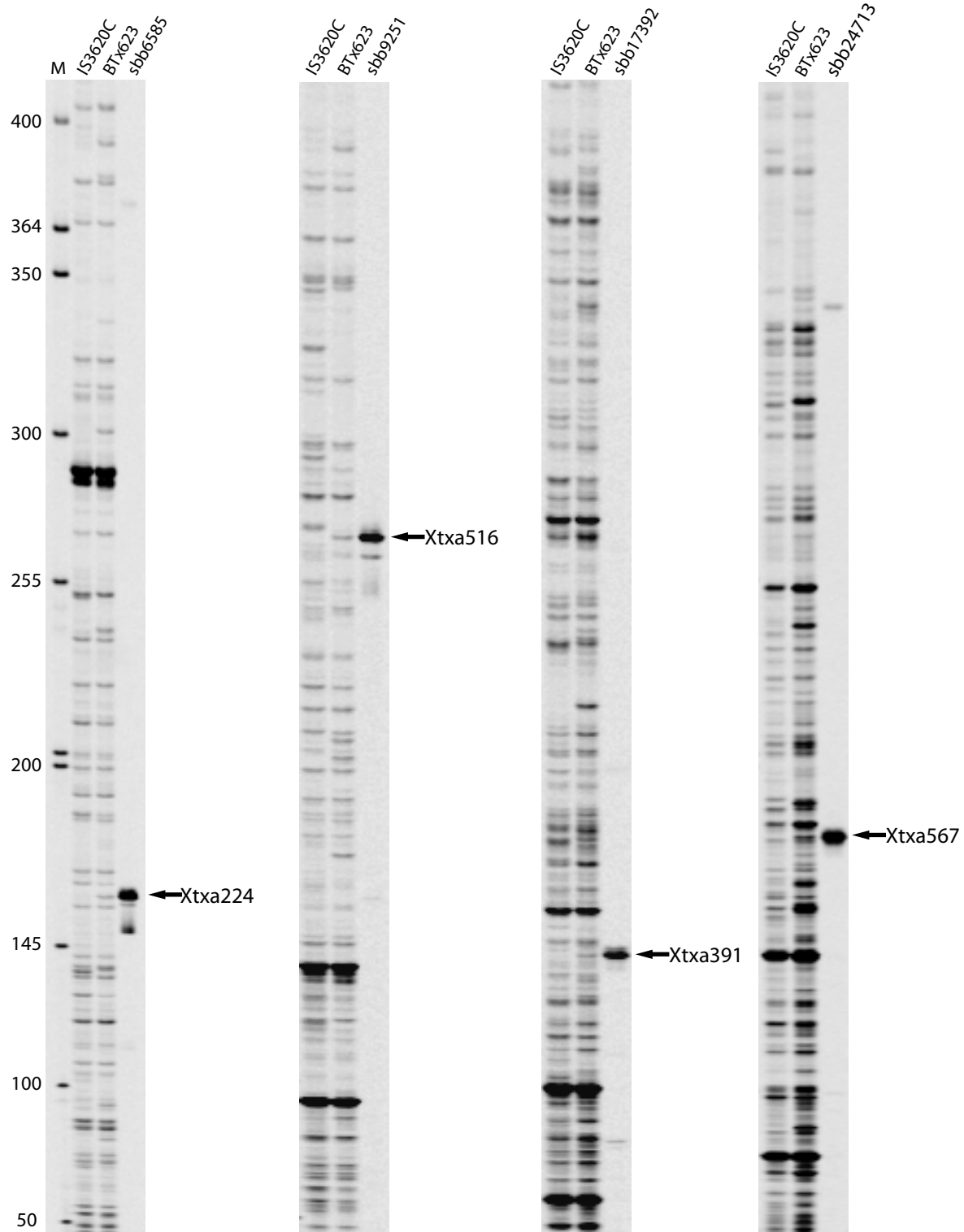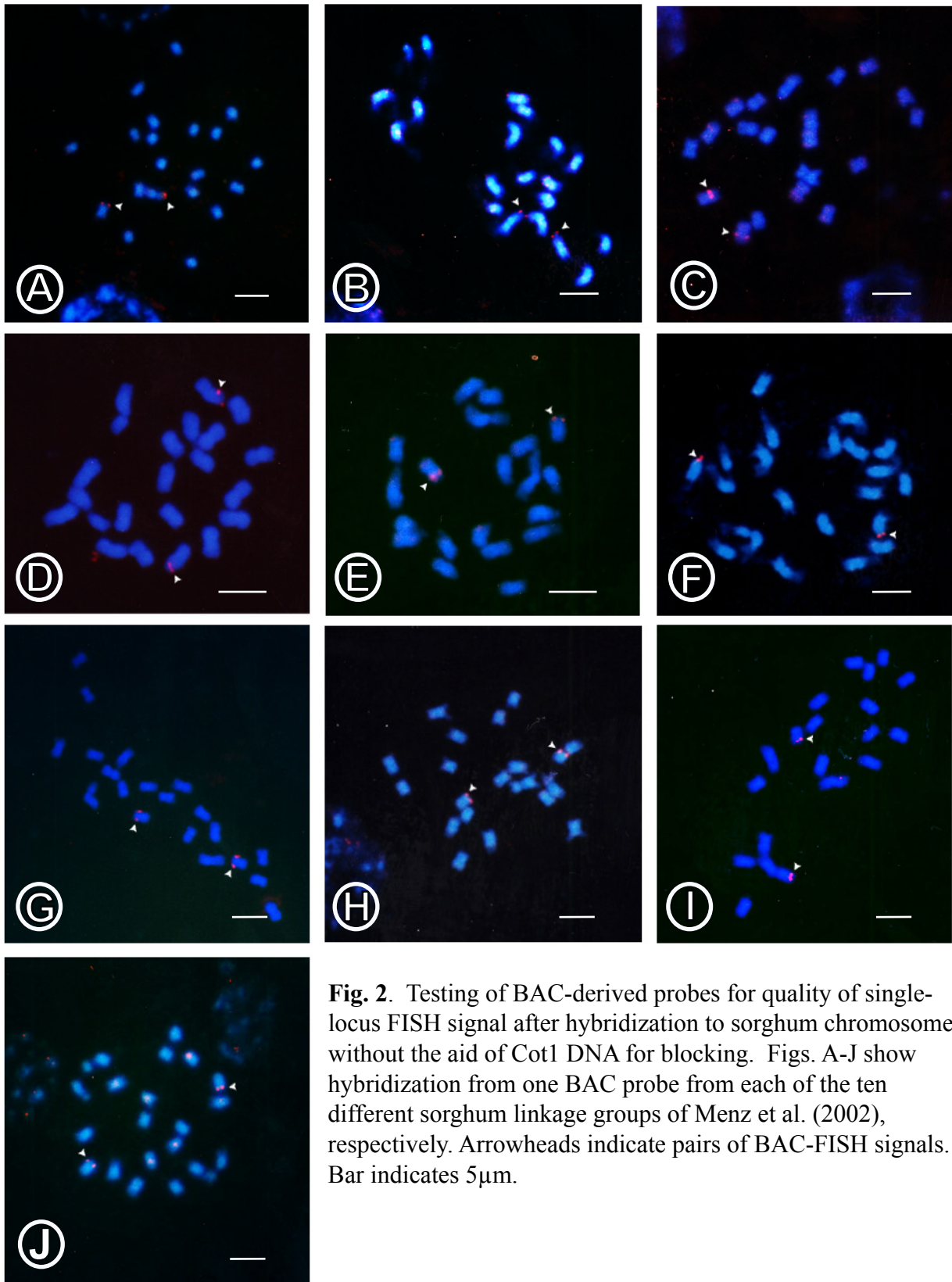
Fig. 2. Testing of BAC-derived probes for quality of single-locus FISH signal after hybridization to sorghum chromosomes without the aid of Cot1 DNA for blocking. Figs. A-J show hybridization from one BAC probe from each of the ten different sorghum linkage groups of Menz et al. (2002), respectively. Arrowheads indicate pairs of BAC-FISH signals. Bar indicates 5μm.

Locations of secondary signals

When FISH results in signals at more than one site, it is important to determine if there is a primary hybridization site, which is usually larger and brighter than the signal at non-primary sites. It is also important to determine that secondary signal sites are constant and represented in both chromatids since that indicates they arose from hybridization based on homology rather than from non-specific binding of the probe or fluorochrome-conjugate. A few of the BACs in this experiment yielded FISH signals at more than one locus. The secondary signals were usually detected on both sister chromatids of both homologues, which discounted origins not dependent on homology.

In contrast with the primary hybridization sites, for which the chromosome identity is known through the linkage group association of the BAC marker, the identity of the chromosome with which such secondary sites associated is not implicitly known unless it happens to be syntenic with the primary BAC-FISH site. Using FISH-based karyotyping, we were able to observe secondary signals on chromosomes other than those bearing the
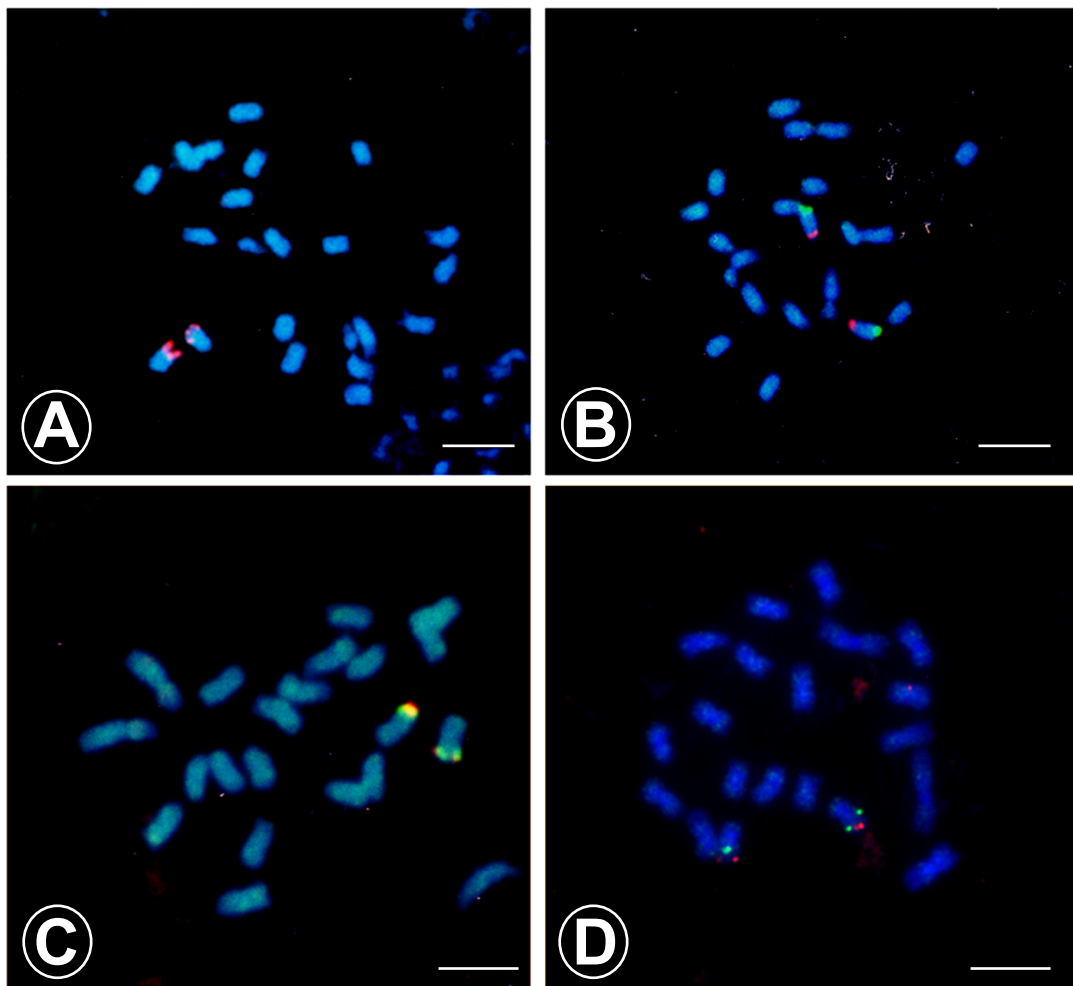
Fig. 3. Two color FISH to test synteny of BACs associated with loci from sorghum linkage groups (Menz et al. 2002). Bar indicates 5µm. (A) Linkage group I. (B) Linkage group B. (C) Linkage group C. (D) Linkage group H.

main signal. For example, the secondary signal of sbb23575, which contains linkage group H marker *Xtxp105*, was found on the chromosome bearing the primary signal of sbb24713, which contains linkage group F marker *txa567* (Fig. 5).

Simultaneous chromosome identification

Based on the above results, probes made from 17 clones distributed across the ten linkage groups were pooled into a BAC-FISH cocktail and used to simultaneously identify all 10 chromosome pairs in two-color FISH on mitotic chromosomes. In the resulting preparations, each chromosome had one, two or three different BAC-FISH loci with various color(s) of signal (Figs. 4A-4D). Using the distribution of pattern and color of signals, all chromosome pairs were readily identified. The relative positions of FISH markers enabled comparisons of physical positions of markers in the chromosomes and linkage maps (Fig. 5). Although several of the BACs (*Xtxp31, Xtxs1563, gap1, Xtxa238, Xtxa567*) were associated with linkage markers that defined interior positions within their respective linkage group, all of the FISH markers except sbb22989 (*Xtxp31*) were located near the ends of chromosomes.

Fig. 4. Figs. A-D are single (DAPI or FITC), dual (DAPI, Cy3) and triple (DAPI, FITC, Cy3) bandpass filter images from a chromosome spread after simultaneous hybridization of 17 sorghum BACs, following single and combinatorial labeling of individual BACs with biotin- and digoxigenin haptens, respectively detected with FITC (green) and Cy3 (red). Pairs of letters denote respective linkage group of the sorghum map (Menz et al. 2002). Solid arrowheads denote BAC probes labeled with both biotin and digoxigenin (1:1). Bar indicates 5μm. (A-D) Photographs from fresh preparations. (A) DAPI counter-stained sorghum chromosomes. (B) Cy3 image showing hybridization signals of eight digoxigenin-labeled BACs and two BACs labeled with both haptens (solid arrowhead). (C) FITC image showing pairs of primary hybridization signals for seven biotin-labeled BACs, one pair of secondary signals for sbb23575 (hollow arrowhead), and two BACs labeled with both haptens (solid arrowhead). (D) Digital image from 3-week old FISH preparation showing hybridization of 17 sorghum BACs. Yellowish color colocalized with strong Cy3 red signals (*e.g.* On A and D) was artifactual, due to the high level digital enhancement used to accentuate the faded FITC signal.
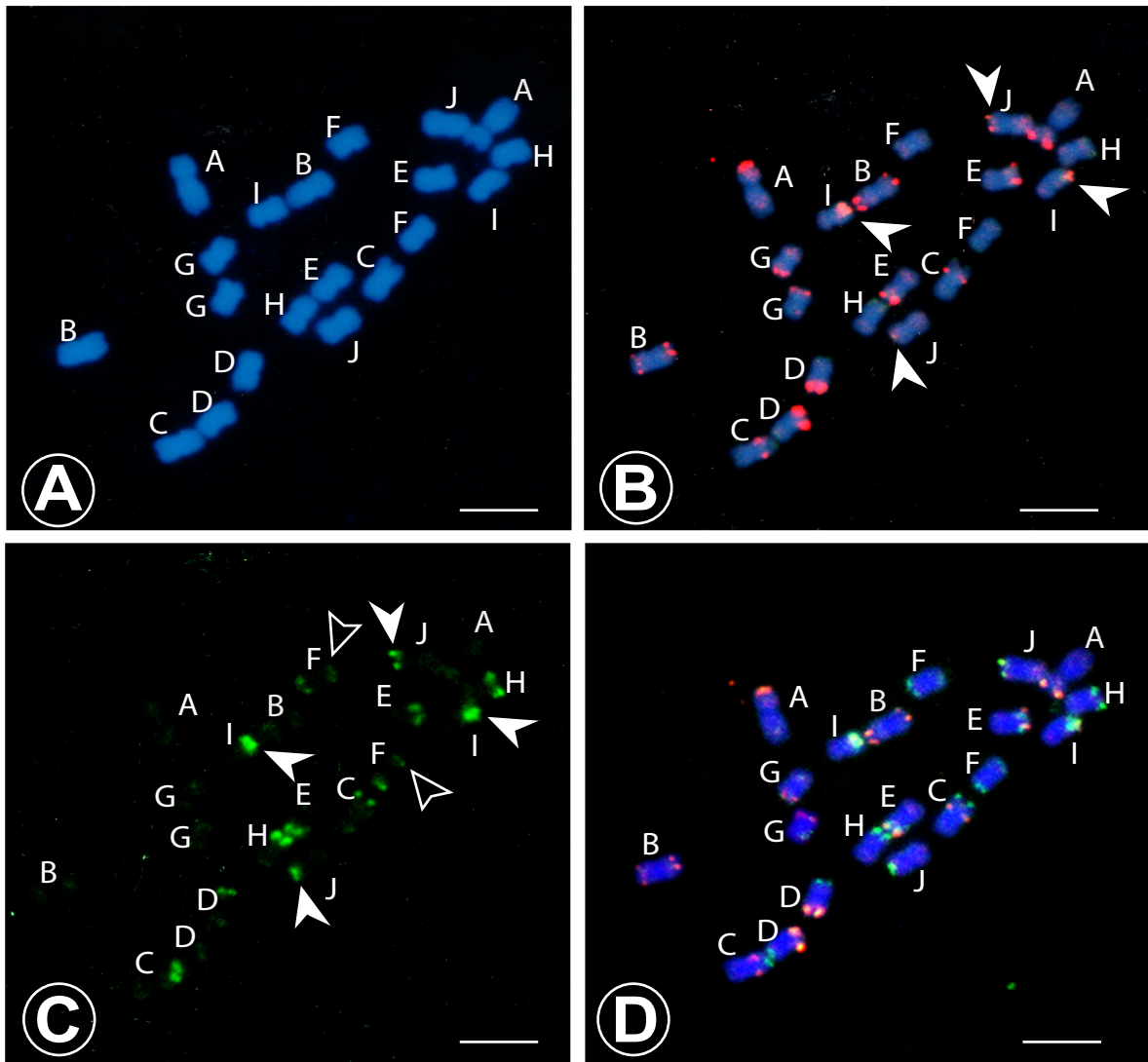
Fig. 5. Comparison of linkage map, chromosome diagram and FISH images. Bar indicates 50cM on linkage maps. (A) Linkage maps, showing a partial set of loci from each linkage group, including the markers used to select BACs for FISH (Menz et al. 2002). (B) Diagrams of chromosomes, where the colored shapes represent signal of BAC clones selected by markers on (A). Green square: FITC detection, Red circle: Cy3 detection, Pink triangle: Combined Cy3+FITC detection. (C-E) Paired images of multi-site, multi-BAC FISH experiment. (C) Digital image from 3-week old FISH preparation showing DAPI, Cy3 and FITC. Signal distributions correspond to individually photographed Cy3 (Fig. D) and FITC (Fig. E) images. In the digital image, the yellowish color colocalized with strong Cy3 red signals was artifactual, due to the high level of digital enhancement used to accentuate the faded FITC signal (green channel). (D-E) Photographs from fresh preparations. (D) DAPI + Cy3 image taken photographically. (E) FITC image taken photographically. Arrowhead at the linkage group F indicates the minor signal of BAC clone, sbb23575 of linkage group H.

Linkage Group A

Xtxa3383
Xtxa224
isu76
umc167
isu165 — sbb6585
Xtxs1225
umc95
PhyB
Xtxa3303

A B C D E

Linkage Group B

Xtxa2851
Xtxp50
Xtxs1891
isu151 — sbb19188
isu117
Xtxa391 — sbb17392
Xtxs1610

A B C D E

Linkage Group C

isu52
Xtxa245
isu91
isu166
Xtxp31 — sbb16368
Xtxs578 — sbb22989
Xtxs1092
Xtxa3395

A B C D E

Linkage Group D

Xtxa2353
Xtxs754
isu56
Xtxp12 — sbb12415
umc54
Xtxs604 — sbb10186
Xtxa516 — sbb9251
umc104

A B C D E

Linkage Group E

Xtxp40
isu139
Xtxp159
isu38
Xtxs1563
Xtxs1554 — sbb9532
Xtxa6102 — sbb11247

A B C D E

Linkage Group F

isu140
umc132
Xtxs1383 — sbb24713
Xtxa567
Xtxs1150
Xtxs2148.1

A B C D E

Linkage Group G

Xtxa2678
isu136
gap1
umc218
rz143.1 — sbb9387

A B C D E

Linkage Group H

Xtxa2948
Xtxp105
Xtxp18
— sbb23575
isu54 — sbb18071
Xtxp47
Xtxp273

A B C D E

Linkage Group I

Xtxp6
cdo718
Xtxa238
Xtxs1906
Xtxp17
Xtxa528 — sbb10491
Xtxa342 — sbb10718

A B C D E

Linkage Group J

isu84.2
Xtxp65
Xtxs722 — sbb12906
Xtxp123
Xtxa2902

A B C D E

Discussion

The facility of large-insert library construction, high quality large-insert clonal DNA isolation, and marker-based clone selection make it highly feasible to develop FISH probes in a targeted and/or large-scale manner. In this study, we targeted each of the 10 chromosomes of *Sorghum bicolor* for development of one or more high-quality FISH probes that would associate each chromosome with one of its ten linkage groups (Chittenden et al. 1994; Kong et al. 2000; Menz et al. 2002). Furthermore, we required probes that could be analyzed by FISH while using only modest or, preferably, no "blocking" with unlabelled repetitive DNA sequences, *e.g.*, Cot1 fraction equivalents. The sensitivity of FISH is highest when blocking DNA is not used.

Most BAC libraries are comprised of genomic clones that are 100-200 Kb in size, and the targets of FISH are thus relatively large and easy to detect. Such large genomic clones, however, are more likely than small clones to contain dispersed repetitive sequences that cause high FISH background signal. With the aim of simultaneously localizing many BACs on individual chromosome spreads, we sought to pick BACs with relatively low repetitive sequence content, and relatively high gene content or at least high unique sequence content. The avoidance of repetitive elements can be critical to the success of BAC FISH. For example, sequence analysis revealed that BAC sbb3766 contains only five regions of repetitive sequence (D.T. Morishige, K.L. Childs, J.E. Mullet, unpublished data). Nevertheless, when this BAC is tested using FISH without suppressive DNA, it produces a painting-like signal over all of the chromosomes. This indicates that even a small amount of certain repetitive sequences in a BAC can result in

significant background signal. In previous studies, we have often used Southern blots of individual BAC DNAs probed with labeled genomic DNA to screen against BACs with abundant repetitive sequences (*e.g.*, Hanson et al. 1995). Here, our approach was indirect. We selected BACs that were identified by molecular markers that mapped to regions of high recombination near the ends of each linkage group of the sorghum linkage map (Menz et al. 2002). Several previous and recent studies have shown that recombination rates are correlated to several features, including high GC content, high gene density and a relatively low density of at least certain repetitive sequences (Gill et al. 1996; Collins et al. 1996; Barakat et al. 1997; Broman et al. 1998). We anticipated that BAC clonal inserts originating from these regions of the genetic map would be rich in low-copy sequence content and lacking in repetitive sequence content. Thus, FISH probes made directly from these BACs were expected to require little or no suppressive hybridization to achieve unambiguous FISH-based localization. Several recent studies have extended the correlation among GC content, gene density, recombination and other features (Barakat et al. 1999; Faris et al. 2000; Gerton et al. 2000; McCombie et al. 2000). Recent work in sorghum using cDNA selection with sorghum BACs has also shown that BACs linked to markers from regions of high recombination tend to be gene rich (Childs et al. 2001), lending support to this simple strategy. Some BACs, *e.g.*, sbb6585, yield one or more pairs of secondary hybridization signals that are less intense than the primary signal sites. The mapping of secondary signals is crucial to molecular cytogenetic applications and also to detection of redundancies within genomes. When such secondary FISH sites are syntenic with the respective primary FISH site, the

chromosomal location is implicit. However, additional information is needed when secondary FISH sites are nonsyntenic with the primary FISH sites. The ability to identify the chromosomal location of secondary BAC-FISH sites is not assured unless the spread is karyotyped. For example, the secondary signal of sbb23575 (linkage group H) was nonsyntenic to its primary signal, but was syntenic to sbb24713 signal (linkage group F). The ability to identify each of the chromosomes across multiple cells by FISH-based karyotyping is expected to facilitate the analysis of the sorghum and related genomes for segmental duplicated segments, polyploidy and repeated sequence distributions.

During the development of linkage maps, the numbers of markers is typically less than desired, and the degree of genomic coverage is uncertain. The degree to which a linkage map provides good coverage can be grossly evaluated by FISH of BACs selected with markers from the ends of linkage groups, as exemplified in bovine with microsatellite selected BACs (de Donato et al. 1999). In sorghum, FISH of BACs selected with markers from the ends of linkage groups B and D yielded FISH signals located at the ends of the respective chromosome arms. The findings indicate that the physical coverage of the chromosomes is nearly complete for linkage groups B and D. The two linkage group I markers *Xtxa238* and *Xtxa528* define a segment that spans over 50% of the BTx623 X IS3620C sorghum linkage map (Menz et al. unpublished), whereas their respective BAC-FISH signals were very near each other on the distal region of the same arm. This reinforces the concept that the recombinationally active regions of sorghum chromosomes are primarily in the large distal euchromatic segments.

The results indicate that linkage and physical maps of sorghum can be used to

facilitate development of an integrated map that includes a chromosomal map of the sorghum genome. The feasibility of identifying sorghum BACs that are highly amenable to simultaneous FISH and FISH-based karyotyping is clearly demonstrated for segments that are gene-rich and high in recombination. Approaches that take advantage of large-insert libraries and integrated genomic resources seem to largely obviate the need of flow sorting or micro-dissection for FISH probe development. Complementary large-insert libraries based on different restriction digests offer excellent coverage (Tao et al. 2001), are amenable to large-scale robotic screening and manipulation, and allow for facile high-quality DNA extraction prior to probe development. In terms of FISH probe development, approaches based on integrated structural genomic resources will allow for targeting, precision and breadth. The approach exemplified herein indicates that development of an extensive molecular cytogenetic karyotyping system for sorghum is highly feasible. When linked directly to linkage and physical (contig) maps, BAC-FISH markers assume greater importance as an integrative genomics resource from which plant research will profit markedly. Moreover, we have shown previously that transgeneric marker-based BAC selection and transgeneric BAC FISH are quite feasible among at least some Gramineae, *e.g.*, rice, sorghum, sugarcane and maize (Zwick et al. 1998). We expect therefore, that probes from a comprehensive molecular system for sorghum are likely to be applicable to other gramineous species, and thus constitute a framework for development of a molecular cytogenetic system for Gramineae. Such a system would facilitate both integrative and comparative genomics among gramineous species.

CHAPTER III

KARYOTYPE NOMENCLATURE, LINKAGE MAP ORIENTATION AND

INTEGRATION ACROSS 36 GENOMIC REGIONS

Introduction

For conventional karyotyping, chromosomes are aligned vertically, grouped by size and centromere location (metacentric, submetacentric, or acrocentric) and they were numbered by size and centromere location. The identification of homologous chromosome pairs is not easy task. Measurements of size are critical, but are subject to effects of preparation. The practical importance of artifacts tends to be greater for small chromosomes, and can lead to changes of relative size for chromosomes similar in size. Chromosomes are flexible structures that condense and elongate during different stages of cell division (Kato and Fukui 1998). In a karyotype, the chromosomes can look bent or twisted and this is a result of how they were sitting on the slide when the photograph was taken. It is also known that chromosome parameters such as relative length and arm ratio may vary even for homologous chromosomes, and thus can lead to disputable or even incorrect conclusions (Matern and Simak 1968; Endo and Gill 1984). The variation on length of the chromosomes caused by artifacts is often much greater than the genetic variation (Matern and Simak 1968). While conventional karyotypes remain valuable, their utility is limited at some point in terms of resolution and variance, especially for small chromosomes that are equal or similar in size, arm ratio. Chromosome banding

methods have unfortunately been far less effective in most plants than in mammals. This has severely limited the utility of conventional karyotypic analysis in development of plant genomics in all but a few species with very large chromosomes.

FISH can be used to detect submicroscopic structure that is beyond the resolution extended banding chromosome studies. Multi-color digital fluorescence microscopy and image analysis is used extensively for the analysis of cytogenetic rearrangements using molecular probes such as cloned DNA fragments and chromosome paints. Since *in situ* hybridization was developed (Gall and Pardue 1969; John et al. 1969), its use has expanded into many fields of genetics and cell biology, *e.g.*, karyotyping (Chen et al. 2000; Dong et al. 2000), genomic introgression (Jacobsen et al. 1995), and physical mapping (de Jong et al. 1999; Sadder et al. 2000).

Gu et al. (1984) analyzed karyotype morphology, including chromosome length, centromere position, arm ratio, secondary and tertiary constriction for seven sorghum species of three *Sorghum* subgenera. Yu et al. (1991) identified every chromosome of Combine Kafir 60, a sorghum cultivar, using a Giemsa C-banding technique in conjunction with chromosome length and arm ratio measurements. Integrated FISH-based identification of sorghum chromosome by *in situ* hybridization of landed BACs was reported by Kim et al. (2002). FISH signals could serve as excellent cytological markers for chromosome or segment identification based on locus-specific FISH signals.

In this experiment, FISH signals were used to identify chromosome pairs easily and unambiguously, and to align and orientate linkage maps relative to the chromosomes. Chromosome lengths, arm lengths and arm ratios were determined. Data were used to

make a standardized karyotype (ideogram) for *Sorghum bicolor* (Moench.), a new nomenclature for chromosomes and linkage groups in contemporary sorghum genomics.

Materials and Methods

Selection of BACs for FISH

BACs anchored by markers from 10 different linkage groups were selected (Klein et al. 2000; Menz et al. 2002). Each BAC was tested for signal quality and when two or more BACs were selected from one linkage group, they were checked on metaphase chromosome for synteny. Only BACs that gave clean one pair of signal were selected for karyotyping.

Plant material and chromosome preparation

Sorghum (*Sorghum bicolor* [L.] Moench.) plants (BTx623) were grown under glasshouse conditions. To induce a high mitotic index, roots of BTx623 plants were trimmed, the plants repotted, and roots harvested two days later. To reduce variation due to differential condensation among mitotic sub-stages, roots were treated with saturated aqueous α-monobromonaphthalene for 2 hr. This extended period of treatment was used to encourage full contraction of euchromatin, and render contraction more uniform across chromosomes and individual mitotic spreads. Excised meristematic tips were placed in enzyme solution (5% cellulase, 2.5% pectolyase in 0.1 M citrate buffer) at

37°C for 40 min, rinsed with distilled water, placed on a clean glass slide with a drop of ethanol-acetic acid (3:1) fixative, macerated using fine-pointed forceps, allowed to air dry at RT for two days, and stored in a –20°C freezer.

In situ hybridization

In situ hybridization techniques were a modification of Jewell and Islam-Faridi (1994) as described by Hanson et al. (1996). The chromosomal DNA on the glass slide then was denatured at 70°C in 100 µl of 70% formamide in 2 × SSC on a hot block for 1.5 min followed by dehydration in 70 at –20°C and 85, 95, and 100% ethanol for each at room temperature. The hybridization mixture (25 µl for one slide) contained 10 ng of labeled probe DNA, 50% formamide, 10% dextran sulfate, 2 × SSC for one–probe FISH. The mixture was denatured at 90°C for 10 min, chilled on ice, and added to the slide. For the hybridization cocktail probed made from 17 different BACs, 50 × *Cot-1* DNA was added. Then the mixture was denatured at 90°C for 10 min, chilled on ice, after which the probe and *Cot-1* DNA were hybridized for 30 min before adding it to the slide. Following overnight incubation at 37°C, slides were rinsed at 40°C in 2 × SSC, 50% formamide in 2 × SSC, 2 × SSC, 4 × SSC plus 0.2% Tween 20 for 5 min each. Slides were blocked 5 min at RT with 5% (w/v) BSA in 4 × SSC plus 0.2% Tween 20. Biotin-labeled probes were detected with 1% Cy3-conjugated streptoavidin and digoxygenin-labeled probes with 1% fluorescein isothiocyanate (FITC)-conjugated anti-digoxygenin antibody. Slides were washed 3 times in 4 × SSC plus 0.2% Tween 20 for 5 min at 37°C.

DAPI in McIlvaine's buffer (9 mM citric acid, 80 mM $Na_2HPO_4^.H_2O$, 2.5 mM $MgCl_2$, pH 7.0) was used for counterstaining chromosomes. After rinsing, Vectashield antifade solution (Vector Laboratories, Inc.) was applied.

Estimation of chromosome size

Images were viewed through an Olympus AX-70 epifluorescence microscope equipped with standard filter cubes. Images from a Peltier-cooled 1.3 M pixel Sensys camera (Roper Scientific) were captured with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, California, USA.). Pairing of homologous chromosomes for karyotypic purposes was processed according to the pattern of signal of each chromosome with aid of MacProbe v.4.2.3. For karyotyped images, DAPI stained chromosomes were measured using Optimas v6.0. The centromere for each chromatid was identified and each arm of the chromatid was measured starting with the short arm and then the long arm. Each chromosome was measured systematically until all the chromosomes had been measured. Average lengths were determined for each arm. The arm ratio (average long arm/short arm ratio), total chromosome length (short arm + long arm), and relative chromosome lengths (length of the individual chromosome/total length of all chromosomes in the genome) were calculated. Data were exported to a spreadsheet (Microsoft Excel) and analyzed.

Results

Chromosome pairing and integration genetic and cytogenetic map

The chromosome number observed in *S. bicolor* BTx623 was $2n = 20$ (Fig. 6). The positions of FISH signals along the chromosomes determined individually for each BAC, using single- or dual-color FISH. When two or more BAC<u>s</u> were analyzed simultaneously, the synteny of their FISH signals was also checked. FISH probes were labeled according to BAC location such that, at detection, a unique pattern of colored FISH signals would identify each type of chromosome. All ten chromosome pairs were identified by signal pattern of 17 different BAC clones anchored by markers from 10 different linkage groups (Table 1 and Fig. 7). Once all chromosome pairs were identified (Fig. 7), each homologous chromosome pair was measured, starting with the short arm and then the long arm. They were numbered according to their rank of the total length with their two arms denoted as S (short) and L (long), respectively (Table 2).

Description of chromosomes

The karyotype of the *Sorghum bicolor* BTx623 consisted of one pair of satellite (SAT) chromosomes (chromosome 1), eight pairs of metacentric chromosomes chromosome 2, 3, 4, 5, 7, 8, 9, and 10, and one pair of submetacentric chromosomes (chromosome 6) (Table 2). Chromosome 1 was the longest (5.11 µm) chromosome. Chromosomes 2, 3, 4, and 5 constituted a group of large chromosomes (3.87 - 3.44 µm), whereas chromosomes 6, 7, 8, 9, and 10 constituted a smaller group (3.15 - 2.97 µm).
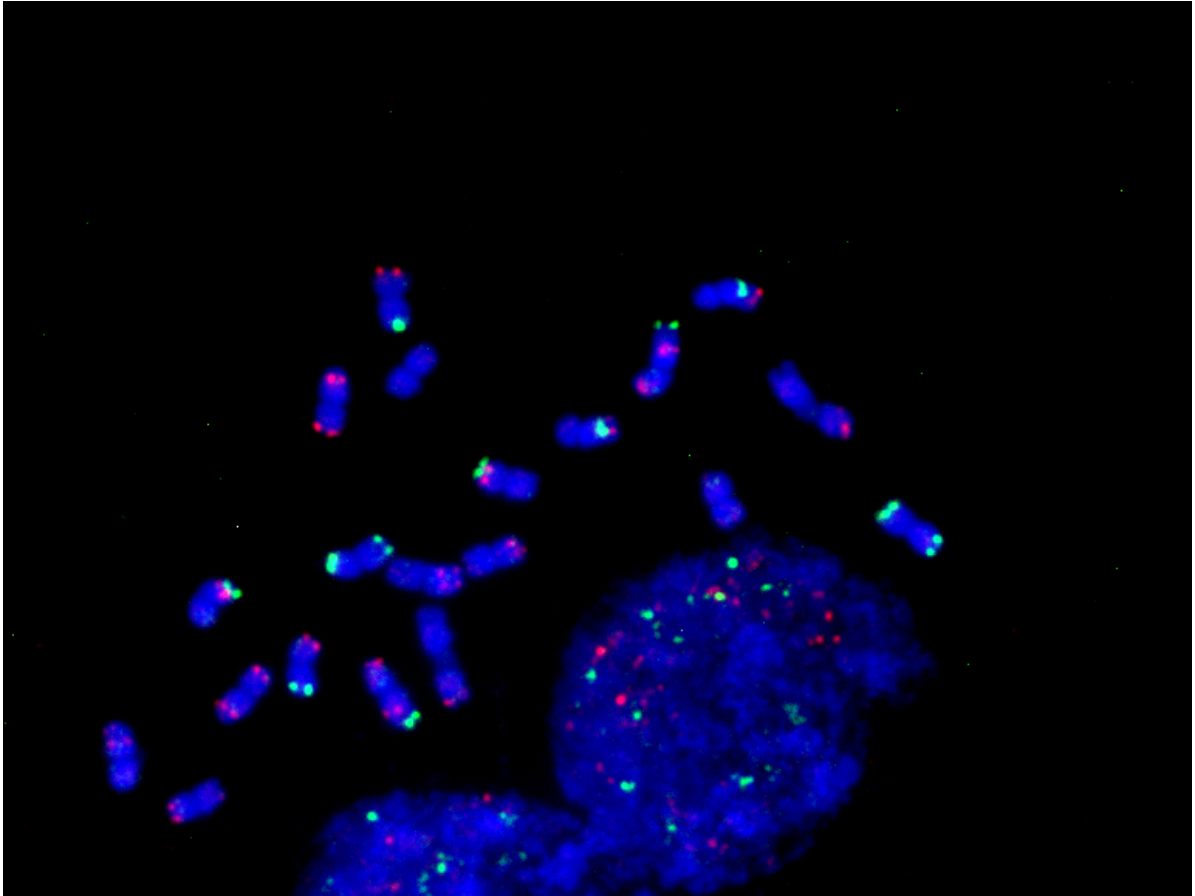
Fig. 6. Simultaneous FISH of 17-BAC cocktail probe to sorghum mitotic metaphase chromosome spread. The patterns of signals enable FISH-based recognition of each chromosome pair and associate specific linkage groups with specific chromosomes (See Fig. 7 karyotype).

Table 1. List of genetic markers and the respective marker-selected BACs used for FISH-based karyotyping.

Linkage group	Marker	BAC (sbb)	Location of signal	Color of signal
A	*Xtxa224*	6585	short arm	red
B	*Xtxp197*	5376	short arm	red
B	*Xtxa6074*	11526	short arm	red
B	*Xtxa4124*	23432	long arm	green
C	*Xtxp31*	22989	long arm	red
C	*Xtxa245*	16368	long arm	red
D	*Xtxs754*	12415	short arm	red
D	*Xtxs604*	10186	long arm	red
E	*Xtxs1563*	9532	long arm	red
E	*Xtxs1554*	11247	long arm	green
F	*Xtxa567*	24713	short arm	red
H	*Xtxp273*	10760	short arm	red
H	*Xtxp18*	18071	long arm	green
I	*Xtxa4032*	9562	long arm	green
I	*Xtxa3926*	25819	long arm	red
J	*Xtxs722*	7043	short arm	green
J	*Xtxa2571*	15070	long arm	green
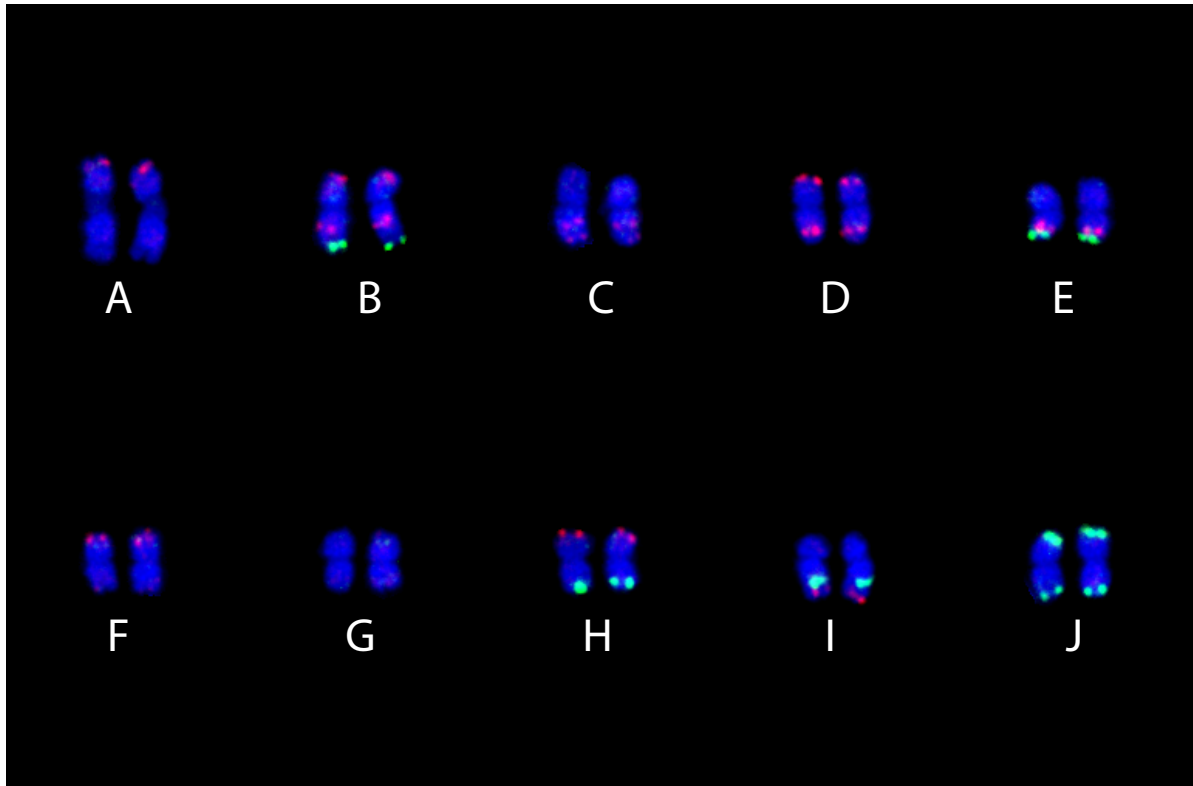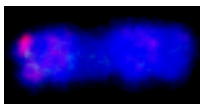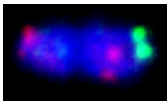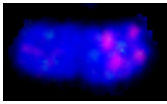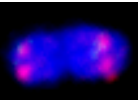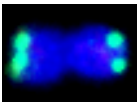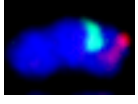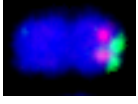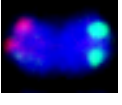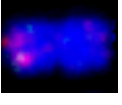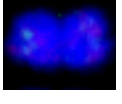
Fig. 7. FISH-based karyotypic pairing mitotic metaphase of sorghum homologous chromosomes in Fig. 6 chromosome spread. Pairing of homologous sorghum metaphase chromosomes based on the color and location of 17 different BAC signals. Each letter corresponds to a linkage group (Menz et al. 2002).

Table 2. FISH-based karyotype of sorghum chromosomes.

Chromosome No.	1	2	3	4	5	6	7	8	9	10
Linkage group	A	B	C	D	J	I	E	H	F	G
Total length (μm)	5.11	3.87	3.85	3.5	3.44	3.15	3.13	3.07	2.98	2.94
Std. error (n=40)	0.047	0.035	0.038	0.032	0.037	0.029	0.028	0.026	0.029	0.023
Relative length (%)	14.59	11.06	10.98	9.99	9.82	9.00	8.92	8.75	8.51	8.39
Arm ratio	1.32	1.16	1.13	1.14	1.02	1.42	1.06	1.10	1.02	1.04

Physical orientation of linkage groups

Chromosome images and positional data were oriented according to cytogenetic convention, i.e., with the short arms at the top vertically aligned chromosomes. Linkage maps were integrated with the cytogenetic map via the markers corresponding to the FISH signals (Fig. 8). The relative orientation of each chromosome in the karyotype was checked against the relative orientation of linkage groups reported by Menz et al. (2002). Chromosomes 1, 2, 4, 5, 6, 7, and 10 were in the same orientation as their respective linkage groups (A, B, J, D, E, I, and G) whereas the karyotypic orientations of chromosomes 3, 8, and 9 were opposite the orientations previously assigned to linkage groups C, H, and F, respectively.

We constructed a detailed chromosome map of *Sorghum bicolor* that integrates the positions of BACs and genetic markers from 36 genomic regions (Fig. 8). A summary of the position of BAC clones and the correlation to the genetic map is given in Table 3 and Figure 8. The physical distances between the loci in cytological map were compared with their distances on the genetic map. It is clear that these distances correlated poorly. Recombination was strongly suppressed in pericentric regions all the 10 chromosomes. For most chromosomes, the centromeric region was located in the middle of linkage and the cytogenetic maps. However, linkage group I had its centromere <19.8% (22.9 cM /

115.8 cM) of the map from the map position 0. That corresponded well to the observation that this linkage group was associated with the only submetacentric chromosome in BTx623, and all other chromosomes are metacentric. The DNA content of each chromosome was estimated using relative length measurements of mitotic chromosomes (Table 2) and the estimated total genomic DNA content, 750 Mbp. The estimated DNA content of each respective chromosome (Mb) or segment was then divided by the length of the respective linkage map (cM) to determine the average amount of DNA per unit recombination (Mb/cM). The ratios for chromosomes, 1, 2, 3, 4, 7, 8, 9, and 10 ranged from 0.40 - 0.47 Mb/cM. Ratios for chromosome 5 and 6, were notably higher -- 0.62 Mb/cM and 0.58 Mb/cM, respectively. In the short arm of chromosome 6, however, a lower recombination rate was observed. The differences in recombination density suggest either suppression of recombination or absence of certain chromosome segments in the genetic map.
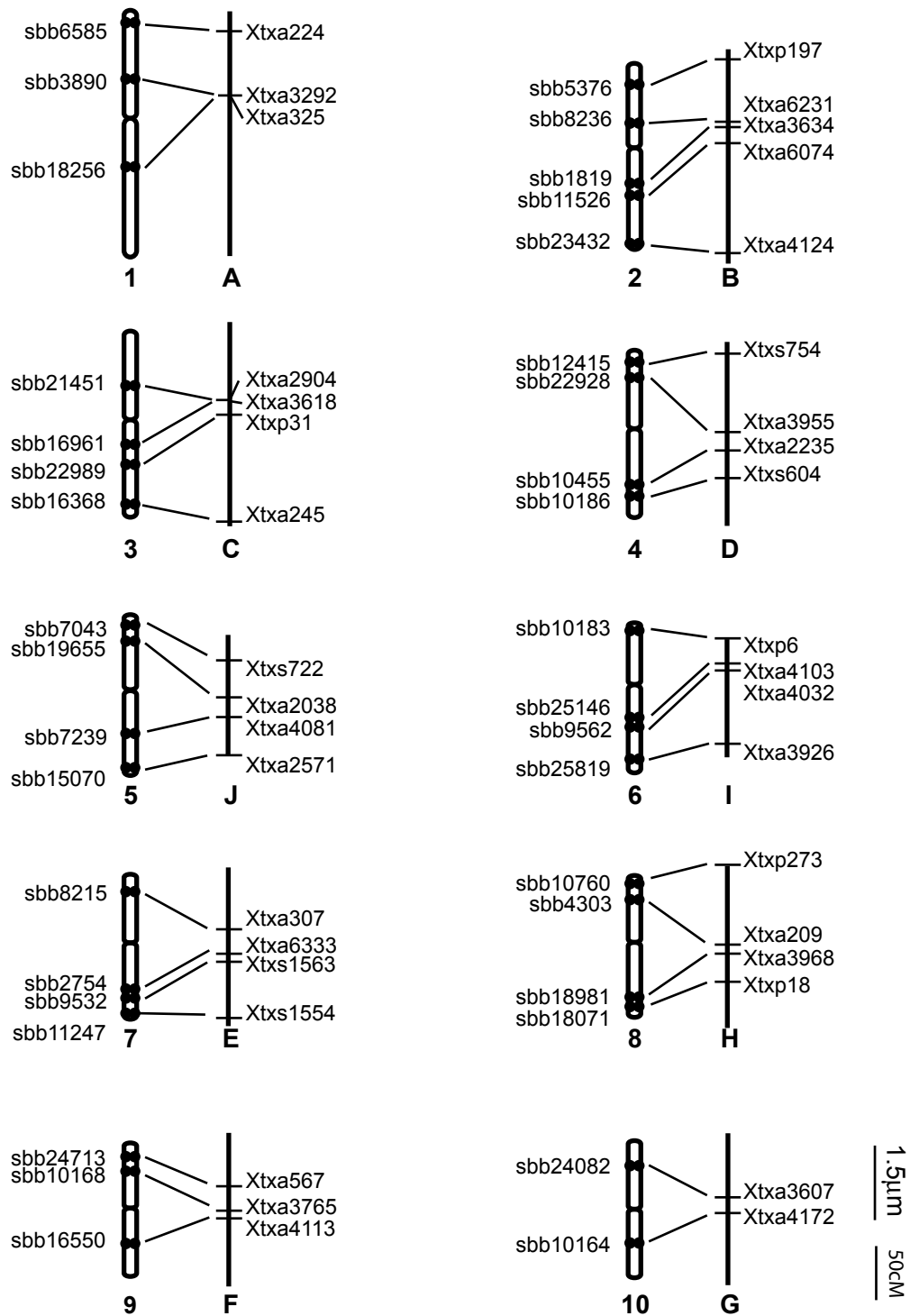
Fig. 8. Correlation between mitotic metaphase chromosomes and linkage groups of sorghum. Chromosomes are numbered according to size and linkage groups are labeled alphabetically. Chromosomes are depicted with the shorter arm in the upper position. BAC clones are positioned on the ideogram according to their positions relative to the centromeres. Bar indicated 1.5μm for metaphase chromosomes and 50cM for linkage maps.

Table 3. List of genetic markers and their anchored BACs used for integrating linkage and cytogenetic maps.

Chromosome map			Linkage map				
Chromosome No.	Arm location of signal	BAC (sbb)	Linkage group	Marker	Map distance	Total map distance (cM)	Average Mb/cM
1	short	6585	A	Xtxa224	16.2-19.1	232.2	0.47
	short	3890		Xtxa3292	75.6-80.5		
	long	18256		Xtxa325	75.6-80.5		
2	short	5376	B	Xtxp197	7.9-11.1	205.2	0.40
	short	8236		Xtxa6231	68.3-71.0		
	long	1819		Xtxa3634	73.3-77.7		
	long	11526		Xtxa6074	89.7		
	long	23432		Xtxa4124	193.4-196.9		
3	short	21451	C	Xtxa2904	76.5-79.9	196.5	0.42
	long	16961		Xtxa3618	76.5-79.9		
	long	22989		Xtxp31	91.0-94.4		
	long	16368		Xtxa245	190.3-196.5		
4	short	12415	D	Xtxs754	9.7	174.6	0.43
	short	22928		Xtxa3955	86.1		
	long	10455		Xtxa2235	100.7-110.6		
	long	10186		Xtxs604	130.1		
5	short	7043	J	Xtxs722	23	118	0.62
	short	19655		Xtxa2038	57.6		
	long	7239		Xtxa4081	80.3		
	long	15070		Xtxa2571	off-end(118)		
6	short	10183	I	Xtxp6	0	115.8	0.58
	long	25146		Xtxa4103	22.9		
	long	9562		Xtxa4032	26.0-29.3		
	long	25819		Xtxa3926	102.3		
7	short	8215	E	Xtxa307	73.9	155.9	0.43
	long	2754		Xtxa6333	85.9-88.0		
	long	9532		Xtxs1563	92.2-97.2		
	long	11247		Xtxs1554	151.2		
8	short	10760	H	Xtxp273	0	152.3	0.43
	short	4303		Xtxa209	72.7-76.4		
	long	18981		Xtxa3968	82.5-86.0		
	long	18071		Xtxp18	109.5-111.2		
9	short	24713	F	Xtxa567	54.4	153	0.42
	short	10168		Xtxa3765	72.8-81.6		
	long	16550		Xtxa4113	85		
10	short	24082	G	Xtxa3607	64.3	148	0.43
	long	10164		Xtxa4172	77.9-80.3		

Discussion

In situ hybridization to mitotic and pachytene chromosomes has been previously
demonstrated to be very useful in plants for assigning linkage groups to chromosomes
(Crane et al. 1993; Reinisch et al. 1994; Fuchs et al. 1998; Dong et al. 2000; Cheng et al.
2001; Kulikova et al. 2001) and for establishing cytogenetic-based physical maps for
single chromosomes and chromosome regions (Zwick et al. 1998; Zhong et al. 1999;
Cheng et al. 2001). Pachytene chromosomes have been used in many studies to obtain a
higher mapping resolution, due to the more decondensed state when compared to mitotic
metaphase chromosomes (Fransz et al. 1998). Pachytene chromosomes clearly display
the differentiation of large heterochromatic blocks around the centromere, whereas the
distal parts of the arms are euchromatic (Fransz et al 2000). On the other hand, a mitotic
metaphase idiogram, in contrast to a pachytene one, allows the estimation of the size of
each chromosome in megabase pairs due to the uniform condensation of chromosomes
at the mitotic metaphase stage (Lapitan et al. 1989) and is therefore useful for
constructing first-generation physical maps.

We have constructed a FISH-based system for karyotyping of sorghum mitotic
chromosomes. The system enabled homologous chromosome pairs to be identified on
the basis of BAC FISH signal position and color. Each pair of FISH signals
corresponded to a specific genetic marker from one of 17 genomic regions. Paired
chromosomes were measured and then ordered according to length. The longest pair of
chromosomes (Chr–1) contained the only nucleolus organizing region (NOR) and was

distinctly larger (5.11 μm) than the other nine pairs. Aside from Chr-1, all mitotic chromosomes were of similar length (2.97 - 3.87 μm) and metacentric, except chromosome 6 (Chr-6), which was submetacentric. The remaining eight sorghum chromosome pairs are relatively uniform and have thus not been very amenable to distinction by conventional karyotyping methods. While crude Giemsa-based C-banding enables the discrimination of all ten pairs of chromosomes, contemporary genomics and comparative cytogenetics require a far more sophisticated approach. Results to date show that FISH enables FISH-based karyotyping of BTx623 standard, and will be an ideal tool for genome-wide integration of cytological and genetic maps of sorghum.

BAC-FISH experiments that involve genetic markers from a total of 36 genomic regions now provides a new integrated chromosome map of *Sorghum bicolor*, and greater coverage of the genome. This extensive correlation of genetic and chromosomal maps in sorghum enables the determination of ratios between physical and genetic distances for numerous segments that collectively represent nearly the entire chromosome complement. In sorghum, higher recombination rates occur toward the chromosome ends. This phenomenon has been demonstrated previously in wheat, barley, and tomato (Tanksley et al. 1992; Gill et al. 1996; Künzel et al. 2000). In *Arabidopsis* and rice, however, recombination hotspots seem to be more randomly distributed and only centromeres are not subject to recombination (Schmidt et al. 1995; Harushima et al. 1998; Arabidopsis Genome Initiative 2000; Cheng et al. 2001). A possible reason for this difference is the additional accumulation of repetitive sequences at proximal chromosome regions with the increase in genome size in most plant species. This is

consistent with the generalized concept of plant chromosome, where genes are clustered

predominantly in euchromatin, most often near chromosome ends (Schmidt and Heslop-

Harrison 1998).

CHAPTER IV

MOLECULAR CYTOGENETIC MAPS OF SORGHUM

CHROMOSOMES 2 AND 8

Introduction

Sorghum is the fifth most important cereal grown worldwide (Doggett 1988). This grain and forage crop is especially important in the semiarid tropics because of its unusual tolerance of hot and dry environment. The relatively small size of its genome (750 million base pairs [Mbp]) (Arumuganathan and Earle 1991) makes sorghum genomics highly complementary to that of rice (440Mb). It will be take advantage of the semi-comprehensive rice genome sequence for sorghum research and comparative analysis of grass genomes. Genome maps enable efficient map-based isolation of genes, targeted genome sequencing, detailed investigation of genome architecture, useful comparison with the genomes of other plants, and association studies that link DNA markers (and genes) to important phenotypes. Discrepancies have been demonstrated between genetic and physical maps, especially in terms of recombination density variation among chromosomal regions (Islam-Faridi et al. 2002; Künzel et al. 2000). Therefore, autonomous linkage maps are poor indicators of molecular sizes and distances. Plant molecular genetic manipulations such as map-aided cloning are thus more soundly undertaken when based on physical maps or linkage maps integrated with physical maps. Rapid progress has made in the construction of an integrated sorghum

genome map. This progress has been achieved using a combination of high-throughput amplified fragment length polymorphism (AFLP) DNA marker technology (Klein et al. 2000; Menz et al. 2002), six-dimensional pooling of bacterial artificial chromosome (BAC) clones (Klein et al. 2000), cDNA capture technology (Childs et al. 2001), and BAC-based fluorescence *in situ* hybridization (FISH) (Kim et al. 2002; Islam-Faridi et al. 2002).

Physical mapping in plants has been achieved by several strategies, including the use of cytogenetic stocks (Weber and Helentjaris 1989; Riera-Lizarazu et al. 2000; Werner et al. 1992; Gill et al. 1993; Künzel et al. 2000), pulsed field gel electrophoresis (Bonnema et al. 1997), BAC or YAC contiguous DNA sequences (Kurata et al. 1997), genomic introgression (Humphreys et al. 1998) and *in situ* hybridization (Jiang and Gill 1994; Stelly et al. 1996) and radiation hybrid mapping (whole-genome radiation hybrid (WGRH) mapping (Walter et al. 1994)). Fluorescence *in situ* hybridization (FISH) is being increasingly applied to physical mapping of plant genomes. Meiotic pachytene chromosomes are much longer than somatic metaphase chromosomes (McClintock 1930), have recently been used as a target for molecular cytogenetic mapping (Wu 1992; Shen et al. 1987; Xu and Earle 1996; Fransz et al. 1998, 2000; Peterson et al. 1999; Zhong et al. 1999; Chen et al. 2000; Song et al. 2000; Islam-Faridi et al. 2002). The construction of physical maps using pachytene chromosomes will deliver directly visible physical evidence of the order and physical position on a chromosome of molecular markers or genes of interest. The comparison of the physical map and the genetic map will enable us to reveal the relationship between physical distance and genetic distance

for the entire sorghum genome. Established correlation between the physical and genetic maps is essential for performing efficient map-based gene cloning and association candidate genes with important biological or agronomic traits.

To integrate linkage maps to cytological chromosome maps in a detailed manner requires multi-point data. The most efficient approach in plants seems to be through FISH of landed BACs, i.e., many BACs that contain specific markers from all along the chromosome. The development of large numbers of FISHable BACs can be a daunting task, because to assess the FISHability of each BAC by FISH is very time-consuming. Here we report the selection of FISHable BAC probes effectively through Southern hybridization.

Materials and Methods

Selection of marker-anchored BACs and BAC DNA purification

The BACs used in this study came from two genomic BAC libraries derived from sorghum cultivar BTx623 (Tao and Zhang 1998; Woo et al. 1994). Markers spaced *ca.* 5 cM apart on each genetic map were used to select BACs. BAC DNA was isolated by alkaline lysis, digested with *Eco*RI, and then further purified using Plant DNeasy spin columns (Qiagen, Valencia, CA) using a modified protocol (Childs et al. 2001).

Southern hybridization

To determine if the amount of sequence repetitiveness in BACs was related to the ability of BACs to yield locus-specific FISH signal without suppression by "blocking" with C_0t-1 DNA, Southern hybridization to BAC DNA was probed with genomic DNA. The results of Southern blots and FISH were compared. BAC DNA isolated by alkaline lysis, digested with *Eco*RI, and then purified using Plant DNeasy spin columns were run into agarose gel and blotted to membrane. The hybridization solution was preheated to 64°C and the membrane was submerged in it and incubated at 64°C for at least 2 hr. Sorghum genomic DNA (20 - 100ng) was denatured at 95°C for 10 min and then placed on ice. Denatured probe labeled with 32P-dCTP using the Klenow fragment of DNA polymerase I at 37°C for >30 min. The labeling reaction product was run on a Sephadex 50G column to separate the free nucleotides from the labeled and non-labeled DNA fragments. DNA was denatured at 95°C for 10 minutes. Denatured probe was transfer into the hybridization solution and incubated at 65°C overnight with gentle agitation. The hybridization solution was discarded and the membrane was washed three times for 15 - 30 min with gently agitated washing buffer that was preheated to 65°C. After removing excessive fluid on the membrane with paper towels, the membrane was wrapped up with Handy Wrap, placed between an x-ray film and an intensifying screen in an autoradiography cassette in a dark room at –80°C. The x-ray film was processed in an x-ray film developer in a dark room.

Pachytene chromosome preparation

Pachytene chromosome preparation was performed according to the protocol of Zhong et al. (1996). Young anthers from Sorghum (*Sorghum bicolor* [L.] Moench.) plants (BTx623) were selected for meiotic chromosome preparations and fixed in ethanol-acetic acid (3:1) fixative. The stage of development was determined from an aceto-carmine squash preparation using a single anther from a flower bud. If at prophase I, the remaining anthers were rinsed in de-ionized water and then incubated at 37°C for 2 hr in enzyme solution (0.3% cellulase, 0.3% pectolyase and 0.3 cytohelicase in 10 mM citrate buffer, pH 4.5), rinsed with distilled water, placed on a clean glass slide with a drop of ethanol-acetic acid (3:1) fixative, macerated using fine-pointed forceps, allowed to air dry at RT for two days, and stored in a −20°C freezer.

***In situ* hybridization**

In situ hybridization techniques were a modification of Jewell and Islam-Faridi (1994) as described by Hanson et al. (1996).

Microscopy

Images were captured using Olympus AX-70 epifluorescence microscope, Peltier-cooled 1.3 M pixel Sensys camera (Roper Scientific) with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, Calif., U.S.A.). To assess relative strengths of FISH signals and their distributions, blue (4', 6-diaminosino-2-2-phenylindole [DAPI] signal from chromosomal DNA), green (FITC from probe) and red

(Cy3 signal from probe) signals were measured from digital images using Optimas v6.0. Lines for sampling luminance values spanned the lengths of somatic chromosomes or meiotic bivalents. Data were extracted for the Optimas v 6.0 "linear morphology default data collection set," exported to a spreadsheet (Microsoft Excel).

Results

Map of chromosome 2

Out of 40 BACs analyzed by single–color FISH, 21 BACs were selected for mapping purposes. To create multi-BAC probe cocktails for simultaneous evaluation of multiple BAC-FISH signals, only BACs which resulted in clean locus-specific signal were selected. Nine BACs were chosen from the short arm of chromosome 2 and twelve from the long arm. Additional BAC probes were developed as part of an iterative process aimed at particular chromosome regions. Given the sequence of their development and the proximity of certain BACs, I constructed two cocktails with overlapping subsets of these BACs for FISH instead of just one complex cocktail (Figs. 9 and 10). Four BACs were common to both probe cocktails, namely sbb10660 and sbb11482 from short arm and sb3867 and sbb11526 from long arm (Figs. 9 and 10). FISH signals on pachytene bivalents revealed that all 21 BACs originated from euchromatic regions. To create diagrammatic representation of cytogenetic location and enable facile comparisons, blue (4',6-diaminosino-2-2-phenylindole [DAPI] signal from

chromosomal DNA), green (FITC from probe) and red (Cy3 signal from probe) signals were measured from digital images using Optimas v6.0, and analyzed using a spreadsheet (Excel). The linear positions of the four common BAC-FISH signals in two figures (Figs. 11C and 11D) matched each other well and facilitated their integrated use. Peak luminance values from BAC–FISH signal were used to assign linear positions along the pachytene bivalent and create a cytogenetic map of chromosome 2 (Fig. 11B). The relative order of BACs was determined by dual-color FISH of pairs of adjacent BACs to pachytene bivalents. The order of individual BAC-FISH loci along the chromosome was fully concordant to that of marker loci along the linkage map (Figs. 11A and 11B). Furthermore, the FISH results resolved the relative order of certain DNA markers previously unresolved by segregation analysis, specifically *Xtxp84* versus *Xtxp50* at one location (25-29 cM), and *Xtxa6252* and *Xtxa3424* at another (141-145 cM).

BAC probe evaluation (BACs from linkage group H)

BAC probe evaluation was performed by Southern hybridization and individual BAC FISH to determine if a useful relationship existed between the pattern of Southern hybridization signal for BACs and that of their FISH signal. Out of 30 >BAC (Table 4) clones tested by FISH, 21 BACs resulted in one clean pair of signals, 5 resulted in a "painting" pattern that is often alluded to as "background" FISH signal, and 4 (sbb14257, sbb23799, sbb26136 and sbb24657) yielded no results. The five BACs (sbb1433, sbb9324 sbb11593, sbb20161 and sbb12305) that yielded strong background signal in FISH experiments also had the strongest signal pattern after Southern hybridization with

genomic DNA probe (Fig. 12). Results indicated that these BAC inserts derived from the pericentromeric heterochromatin region, except the fifth clone (lane 29 sbb12305), which localized to a largely euchromatin region. BACs that yielded only moderate band of signal after the Southern hybridization (sbb10760, sbb18861, sbb9171, sbb7724, sbb24521, sbb16523, sbb10990, sbb20161, sbb18981, sbb23303, sbb18578, sbb12329, sbb19883, sbb18071, sbb14482, sbb23575, sbb16700, sbb66E20, sbb14774, sbb10453, sbb2887) yielded good clean signal on the FISH experiment.

Map of chromosome 8

The 21 BACs that yielded singe-locus specificity (low background) after single-probe FISH were checked for synteny and order. Signals from BACs sbb14774 and sbb18861 went to chromosome(s) other than chromosome 8. The relative order and synteny of 19 BACs on the cyto-molecular map were determined by dual-color FISH of

Fig. 9. FISH signals on sorghum chromosome 2 pachytene bivalent using a 15-probe cocktail. The identity of each individual BAC clone used in the probe cocktail is given next to its representative FISH signal.
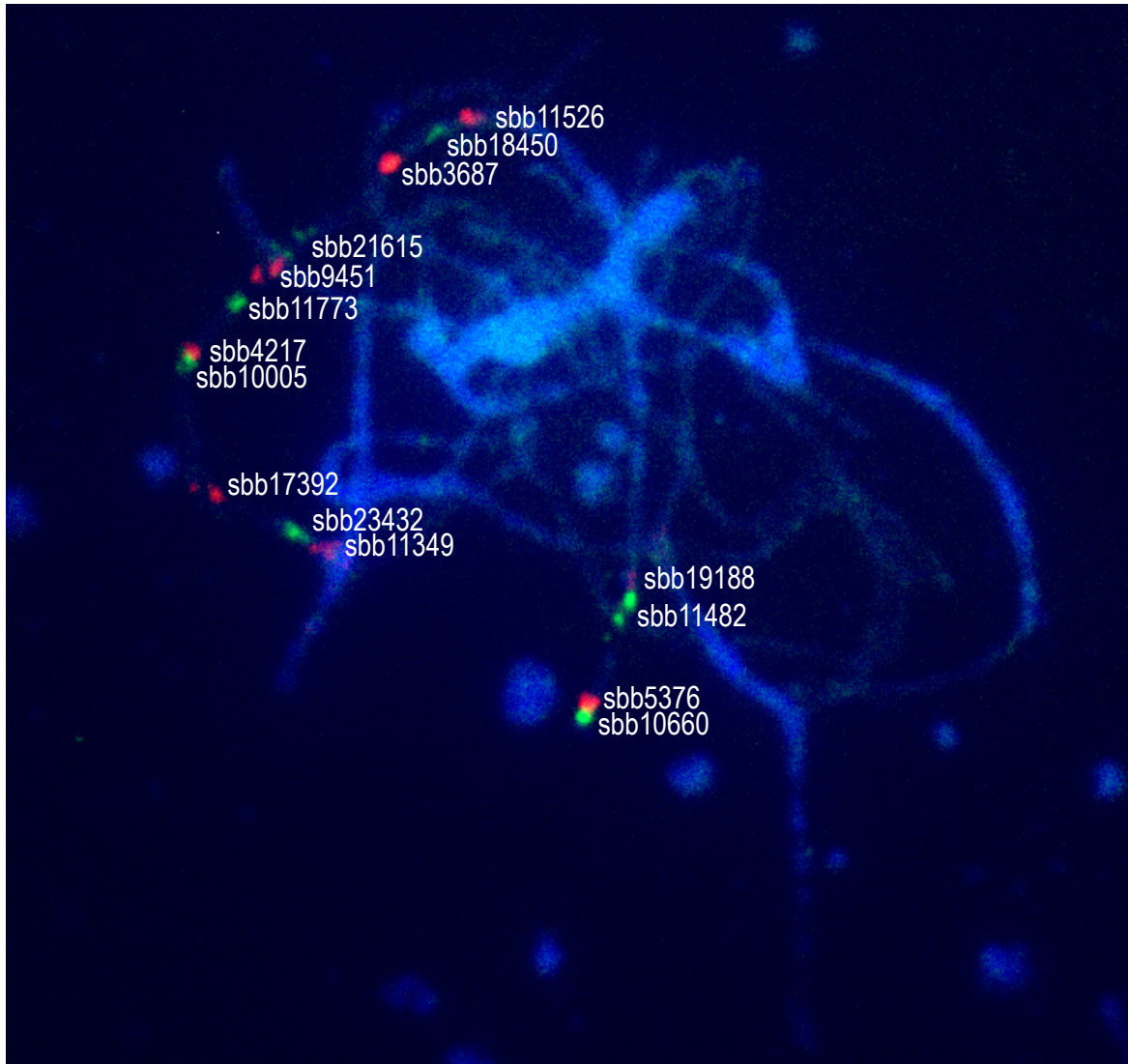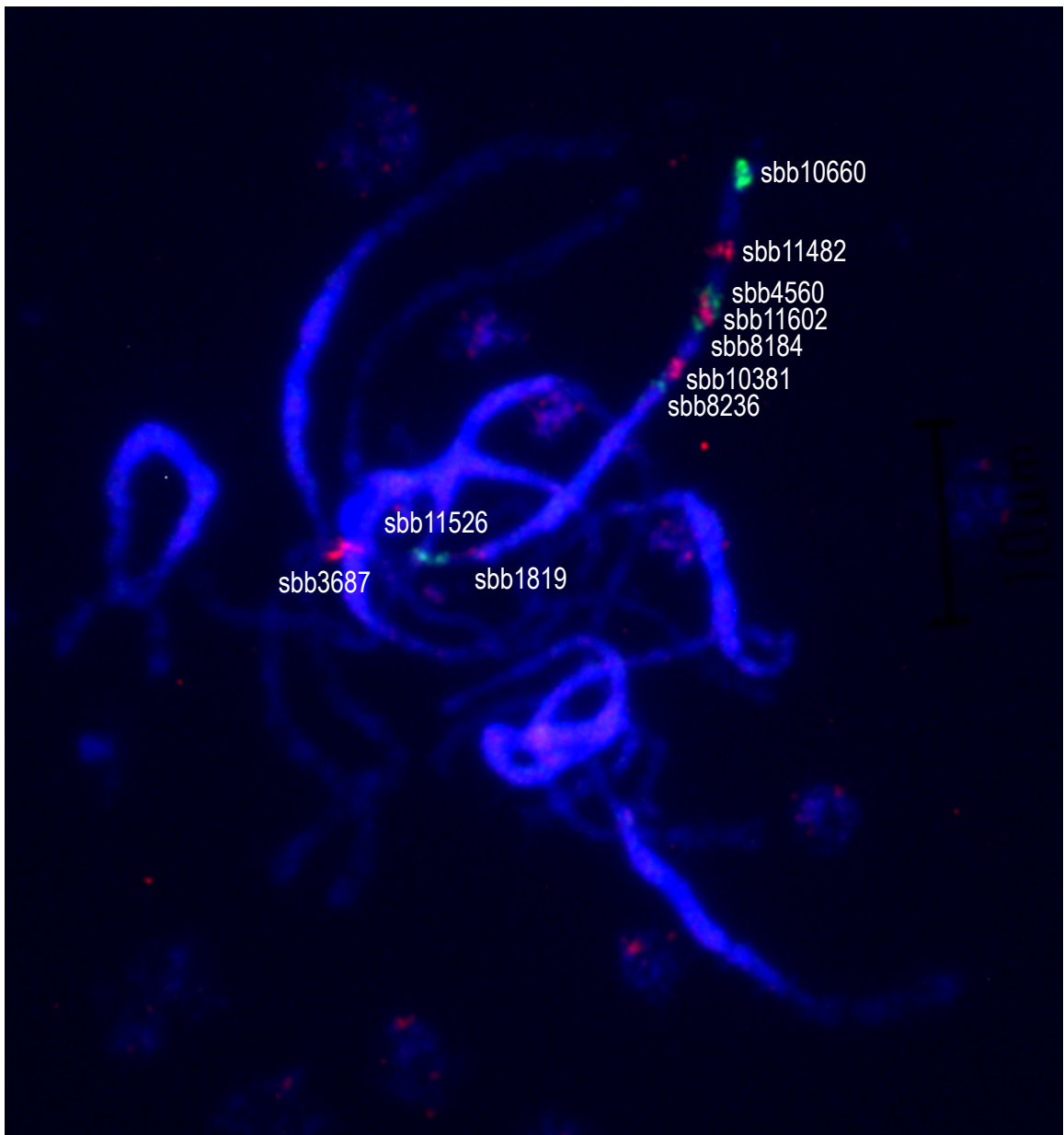
Fig. 10. FISH signals on sorghum chromosome 2 pachytene bivalent using a 10-probe cocktail. The identity of each individual BAC clone used in the probe cocktail is given next to its representative FISH signal.

Fig. 11. A diagrammatic representation of the cytogenetic locations of 21 sorghum BACs on sorghum chromosome 2 and the corresponding marker positions (Menz et al. 2002). (A) Linkage group B markers used to select BACs for FISH (Menz et al. 2002). (B) Diagrams of chromosome 2 pachytene bivalent indicating the positions of signals from BAC that contain LG-B marker loci. (A). Green circles: FITC detection, Red boxes: Cy3 detection. Signals were located based on position of signal peaks from Fig. C. (C-D) Estimation of the strength and location of BAC probes (C for Fig. 9 and D for Fig. 10). Graph peaks represent relative strengths of FISH signals and their distributions; blue (4',6-diaminosino-2-2-phenylindole [DAPI] signal from chromosomal DNA), green (FITC from probe) and red (Cy3 signal from probe) signals. Dashed lines indicate peaks for probes located in both of Figs. C and D. (C) Green probes included sbb10660, sbb11482, sbb18450, sbb21615, sbb11773, sbb10005, sbb23432; red probes included sbb5376, sbb19188, sbb11526, sbb3687, sbb9451, sbb4217, sbb17392, sbb11349. (D) Green probes included sbb10660, sbb4560, sbb8184, sbb8236, sbb11526; red probes included sbb11482, sbb11602, sbb10381, sbb1819, sbb3687. Arrow-head indicates saturated signal from DAPI and arrows indicate signals of DAPI from overlapped parts of the pachytene bivalents.

A	B	C	D

A (genetic map positions):

- 7.5 — *Xtxp63*
- 7.9-11.1 — *Xtxp197*
- *Xtxp84*
- 25.4-29.9 — *Xtxp50*
- 42.5 — *Xtxa2717*
- 48.4 — *Xtxa3451*
- 52.2 — *Xtxa280*
- 64.8-68.3 — *Xtxp4*
- 68.3-71.0 — *Xtxa6231*
- 73.3-75.7 — (CEN)
- *Xtxa3634*
- 89.7 — *Xtxa6074*
- 101.1 — *Xtxa6138*
- 114.8-118.5 — *Xtxa3803*
- 134.1 — *Xtxa6076*
- 141.1-145.2 — *Xtxa6252* / *Xtxa3424*
- 154.6-157.5 — *Xtxp100*
- 160.8 — *Xtxs1845*
- 180.5-184.2 — *Xtxa391*
- 193.4-196.9 — *Xtxa4124*
- 205.2 — *Xtxs1610*

B (markers):

- sbb10660
- sbb5376
- sbb11482
- sbb19188
- sbb4560
- sbb11602
- sbb8184
- sbb10381
- sbb8236
- sbb1819
- sbb11526
- sbb18450
- sbb3687
- sbb21615
- sbb9451
- sbb11773
- sbb4217
- sbb10005
- sbb17392
- sbb23432
- sbb11349

pairs of adjacent BACs to pachytene chromosome. Based on the above results, we used probes from 19 BAC clones anchored to LG-H markers to create a multi-BAC FISH probe cocktail that would simultaneously hybridize to positions corresponding to the BAC genomic DNA inserts. On spreads of pachytene bivalents, FISH signals were readily resolved for each of 19 BAC components in the multi-BAC probe cocktail (Fig. 13). All signals were located in euchromatic regions (Fig. 13). Twelve BACs hybridized to the long arm, whereas seven BACs hybridized to the short arm of chromosome 8 (Fig. 13). For diagrammatic representation of cytogenetic location, blue (DAPI signal from chromosomal DNA), green (FITC from probe) and red (Cy3 signal from probe) signals were measured from digital images using Optimas v6.0, and analyzed using a spreadsheet (Excel). Peak luminance levels of the appropriate color (green or red) represented the location of the corresponding FISH signal and thus the respective BAC and LG marker position. Linear positions of the appropriate peak luminance levels value were used to create a cytogenetic map of chromosome 8, including marker loci and a few key chromosomal features (Fig. 14B). The order of individual BAC-FISH loci along the chromosome was fully concordant to that of marker loci along the linkage map (Figs. 14A-14B). We also were able to resolve the relative order of two sets of DNA markers that has previously not been resolved by segregation analysis (*Xtxs2065* and *Xtxa388*).

Table 4. List of BACs used for FISH, their associated markers, and location on linkage group H. Clone numbers correspond to the number on the Fig. 12. Right-most column – P = clones selected for the probe cocktail and other chr = clones located in other chromosomes than chromosome 8. Column of FISH results - G = good locus specificity, * = high background signal and ? = not determined.

LG	Marker	cM Distance	BAC clone No.	BAC (sbb)	FISH results	BACs used in probe cocktail
H	*Xtxp273*	0	1	10760	G	P
H	*Xtxa3525*	6.1-13.1	2	18861	G	other chr
H	*Xtxa3686*	20.1-22.9	3	9171	G	P
H	*Xtxp47*	38.7	4	7724	G	P
H	*Xtxa4117*	45.5	5	24521	G	P
H	*Xtxa3638*	51.0-55.3	6	16523	G	P
H	*Xtxa3682*	63.8-68.1	7	10990	G	P
H	*Xtxa6081*	72.7-76.4	8	4303	G	P
H	*Xtxa2864*	77.5-80.2	9	1433	*	
H	*Xtxa3667*	77.5-80.2	10	9324	*	
H	*Xtxa2711*	72.7-80.2	11	11593	*	
H	*Xtxa174*	72.7-80.2	12	20161	*	
H	*Xtxa3968*	82.5-86.0	13	18981	G	P
H	*Xtxa3856*	86	14	23303	G	P
H	*Xtxa73*	88.2-90.7	15	23799	?	
H	*cdo459*	99.2	16	18578	G	P
H	*Xtxa388*	99.2-104.2	17	12329	G	P
H	*Xtxs2065*	99.2-104.2	18	19883	G	P
H	*Xtxa2582*	104.2-109.5	19	26136	?	
H	*Xtxp18*	109.5-111.2	20	18071	G	P
H	*Xtxa606*	111.6-113.4	21	14482	G	P
H	*Xtxa3827*	116.6-122.7	22	24657	?	
H	*Xtxp105*	126.1	23	23575	G	P
H	*Xtxa6107*	129.4-132.9	24	16700	G	P
H	*Xtxa6346*	132.9-136.8	25	66E20	G	P
H	*Xtxa588*	136.8	26	14774	G	other chr
H	*Xtxa3876*	140.4	27	16555	?	
H	*Xtxa4024*	143.3-146.3	28	10453	G	P
H	*Xtxa2332*	147.1	29	12305	*	
H	*gap34*	147.1-149.4	30	2887	G	P
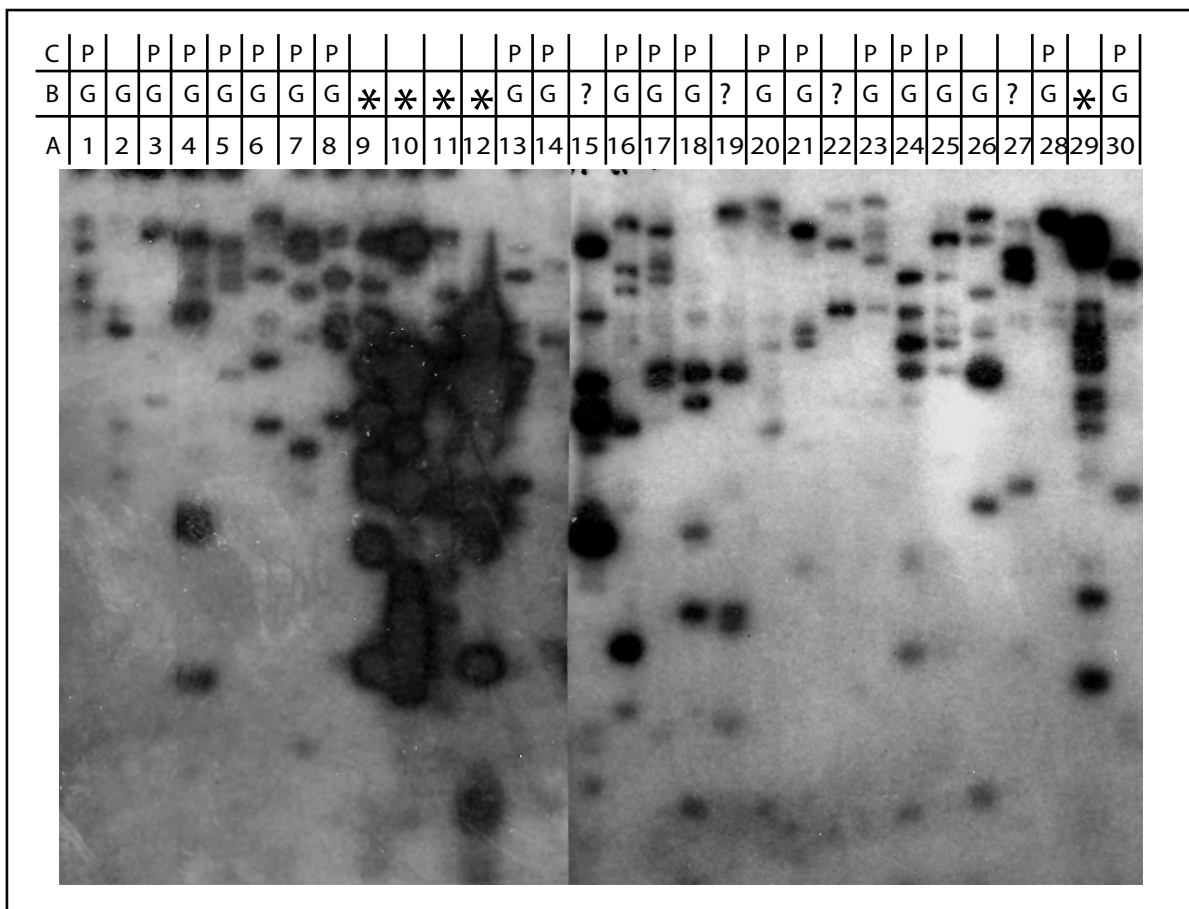
Fig. 12. Southern hybridization result of BACs from Table 4 probed with sorghum genomic DNA. (A) Lane number corresponding to the BAC clone number in Table 4. (B) FISH results - G = good locus specificity, * = high background signal and ? = not determined. (C) BACs used to construct chromosome specific multi-BAC probe cocktail.

Fig. 13. FISH signals on sorghum chromosome 8 pachytene bivalent using a 19-probe cocktail. The identity of each individual BAC clone used in the probe cocktail is given next to its representative FISH signal.

Fig. 14. A diagrammatic representation of the cytogenetic locations of 19 sorghum BACs on sorghum chromosome 8 and the corresponding marker positions (Menz et al. 2002). (A) Linkage group H markers used to select BACs for FISH (Menz et al. 2002). (B) Diagrams of chromosome 8 pachytene bivalent indic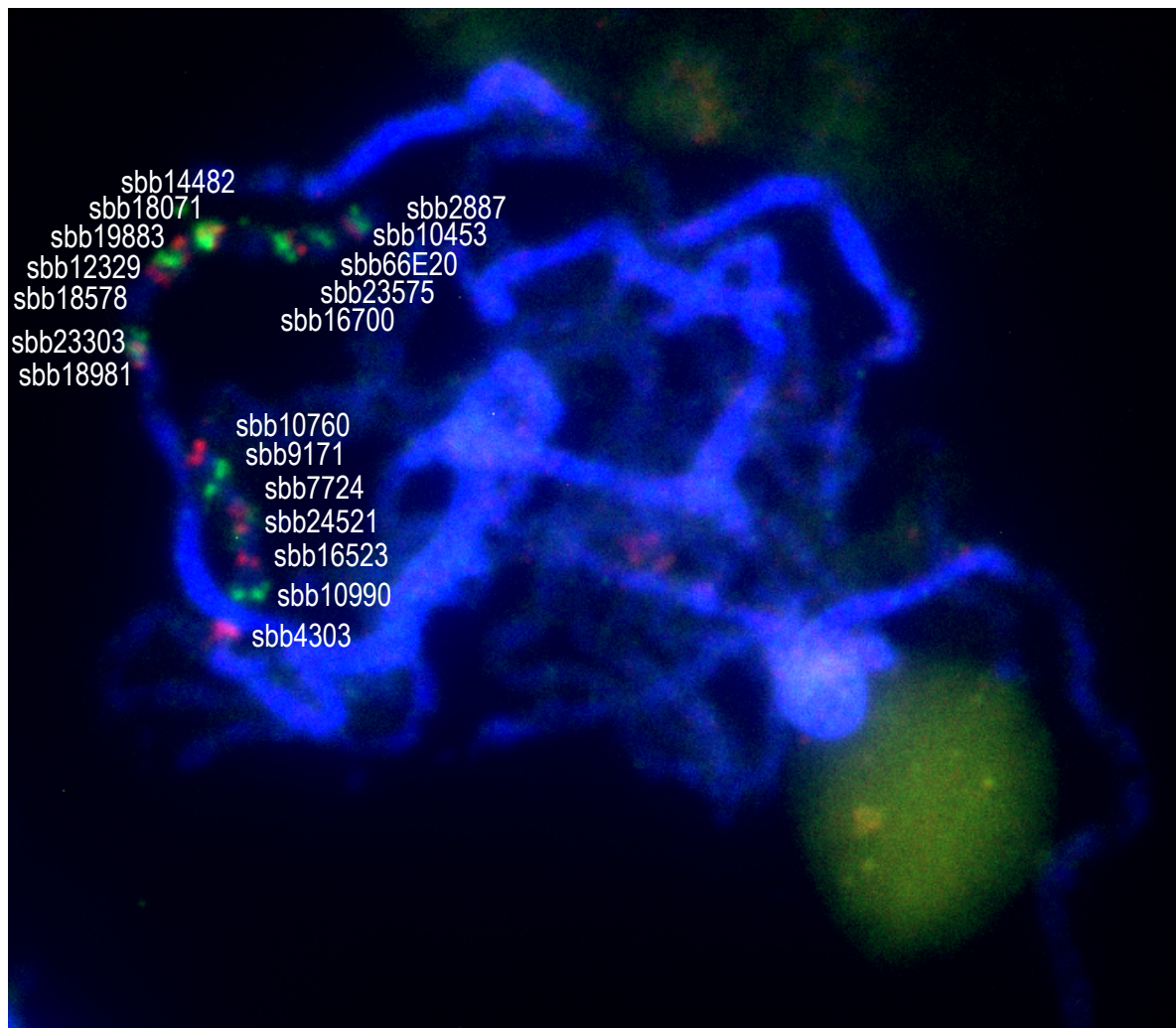ating the positions of that contain LG-H marker loci. (A). Green circles: FITC detection, Red boxes: Cy3 detection. (C) Estimation of the strength and location of BAC probes for Fig. 13. Graph peaks represent relative strengths of FISH signals and their distributions; blue (4',6-diaminosino-2-2-phenylindole [DAPI] signal from chromosomal DNA), green (FITC from probe) and red (Cy3 signal from probe) signals. Arrow-head indicates saturated signal from DAPI and arrow indicates signals of DAPI from overlapped parts of the pachytene bivalents.
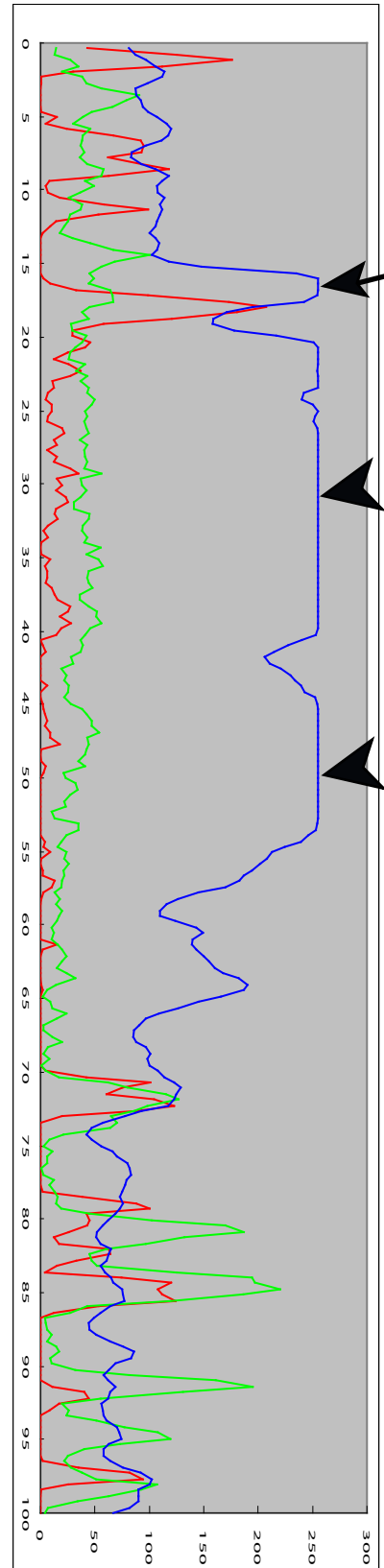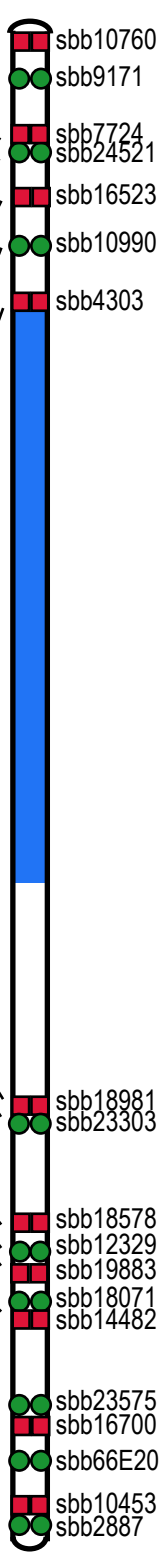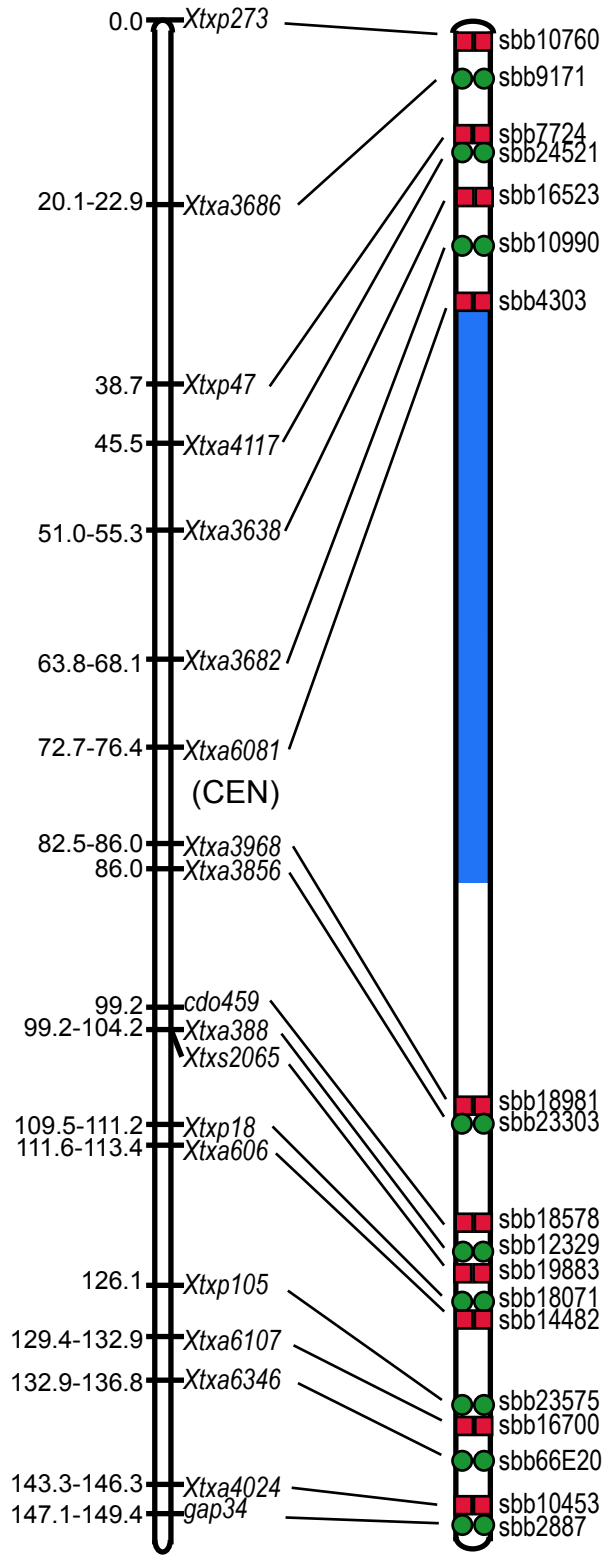
A   B   C

Physical coverage of chromosomes 2 and 8 by linkage groups B and H

Physical positions from BAC-FISH signals were compared to linkage group map positions of corresponding molecular marker loci. BAC clone sbb10660 was shown to contain LG–B marker *Xtxp63* (mapped to 7.5 cM / 205.2 cM) and its genomic insert was revealed by FISH to have originated from very near the end of the short arm. The segment distal to *Xtxp63* was about 1% of the total chromosome–2 bivalent length. BAC clone sbb11349 contains LG–B marker *Xtxs1610* (mapped to 205.2 / 205.2 cM) (Menz et al. 2002), and its insert was shown by FISH to have originated from the end of long arm, where the distal segment is only about 1% of the total chromosome–2 bivalent length. BAC clones sbb10760 and sbb2887 contain LG–H markers *Xtxp273* and *gap34*, respectively, which Menz et al. (2002) mapped to positions 0 cM and 147.1-149.4 cM, where the overall length of the linkage group was 152.3 cM. The corresponding FISH signals were very near opposing ends, with distal segments being just 1.2% and 1.6% of the total length of chromosome 8 bivalents. The results indicate that linkage maps LG-B and LG- H provide excellent coverage of chromosomes 2 and 8, respectively. The overall recombination rates were similar the two chromosomes. The mean rate observed for chromosome 2 was 0.40 Mb/cM, whereas that for chromosome 8 was 0.43 Mb/cM, and the genomic average was 0.46 Mb/cM.

Delimitation of the centromere on the linkage map

Linkage group B (Chromosome 2)

The location of centromere in the LG–B map was determined by observing the locations of FISH signals relative to pachytene bivalent pericentromeric heterochromatin after FISH of BACs that contain LG–B marker loci. Signals from BAC-FISH of sbb8236 revealed that LG–B marker *Xtxa 6231* (mapped to 68.3 - 71.0) is in the short arm near the heterochromatin-euchromatin junction. Signal from FISH of BAC sbb1819 indicated that *Xtxa 3634* (mapped to 73.3 - 75.7) is in the long arm, near the heterochromatin-euchromatin junction of the chromosome 2. The interval defined by markers *Xtxa 6231* and *Xtxa 3634* spans *ca.* 2.4% of LG–B (4.85cM / 152.3 cM), whereas the corresponding heterochromatic segment defined by BACs sbb8236 and sbb1819 accounts for *ca.* 22% of the physical length of the pachytene bivalent (Fig. 12).

Linkage group H (chromosome 8)

The BAC FISH signals corresponding to *Xtxa6081* occurred in the short arm near the heterochromatin-euchromatin junction, whereas BAC-FISH signal for *Xtxa3968* occurred in the long arm little toward to the euchromatic region. Although the interval defined by markers *Xtxa6081* and *Xtxa3968* spans only 6.3% of the linkage group H (9.7 of 152.3 map units), the corresponding physical segment defined by BACs sbb4303 and sbb18981 accounts for >50% of the physical length of the pachytene chromosome 8 (Fig. 14).

A comparison of the physical spacing between BAC-FISH signals on chromosome 2 and 8 bivalents versus spacing of the respective marker loci on the linkage maps reveals that the frequency of recombination differs widely among chromosome regions (Figs. 12 and 14). Most notably, recombination was much lower and thus linkage much stronger across the pericentromeric heterochromatic regions of both chromosomes.

Discussion

Significant discrepancies have been reported between genetic and physical distances in genomes of a number of plant species. In wheat and barley, the genomes are large (*ca.* 5,000 Mb per base genome) and the chromosomes are large (*ca.* 700 Mb/chromosome), and recombination occurs mainly along the distal half of each chromosome arm. Recombination is essentially suppressed in the centromeric regions, which may account for as much as 50% of the length of each chromosome (Werner et al. 1992; Gill et al. 1993; Delaney et al. 1995a, 1995b; Künzel et al. 2000). Variation in recombinational density and structural rearrangements significantly constrains the utility of linkage maps. Upon integration with each other, physical and linkage maps become synergistically useful. Linkage maps become more effective for molecular genetic manipulation, and physical maps become more effective for recombination-based genetic manipulation.

Several molecular marker-based genetic linkage maps of sorghum genomes have been constructed (*e.g.*, Peng et al. 1999; Bhattramakki et al. 2000; Kong et al. 2000;

Menz et al. 2002).   Recently, two key steps were taken toward integration of sorghum linkage and physical maps.   First, Kim et al. (2002) establish a skeletal relationship between linkage group markers and each of the 10 *Sorghum bicolor* chromosomes. Second, Islam-Faridi et al. (2002) established the first high-resolution integrated map for a single *Sorghum bicolor* chromosome**.**

FISH-based physical mapping will be a valuable complement to other genome projects.   The chromosomal locations of uncertain BAC clones or contigs can be unambiguously assigned to specific chromosomes by FISH analysis with the aid of chromosome-specific FISH markers (Cheng et al. 2001).   Pachytene FISH can be used to determine if the linkage gaps represent recombination hot spots or large chromosomal segments.   Such information is valuable in the selection of strategies to close gaps. Pachytene FISH and fiber-FISH can be used to estimate the physical distance between BAC clones.

In this study, BACs were hybridized to pachytene bivalents to assign their cytological location on chromosomes 2 and 8.   FISH of BACs that contain marker loci of linkage groups B and H yielded signals on chromosomes 2 and 8, respectively.   The physical order of FISH signals was fully concordant with the recombination-based maps. Therefore, FISH analysis confirmed the relative order of the DNA markers that had been established through segregation analysis.

Results from BAC-FISH indicated that chromosomes 2 and 8 are well covered by linkage groups LG–B and LG–H of Menz et al. (2002). Although LG–B marker *Xtxp63* mapped to 7.5 cM and thus was not the most terminal marker, the segment of pachytene

bivalents distal to BAC sbb10660 was less than 2% of overall bivalent length.   At the opposite end, less than 1% of the bivalent was distal to FISH signal from BAC sbb11349, which contains the LG–B terminal marker *Xtxs1610* (Menz et al. 2002). In the case of linkage group H, BAC clone sbb2887 contained marker *gap34* (mapped to 2.9 cM), and BAC clone sbb10760 contained *Xtxp273* (mapped to 152.3 cM). FISH signals from sbb2887 and sbb 10760 were very close to opposite ends of the chromosome 8 pachytene bivalent. Segments distal to the FISH signals were just 1.2% and 1.6% of total bivalent length, respectively. The data indicate that genetic linkage maps LG–B and LG-H cover more than 96% of chromosomes 2 and 8.

The positions of centromeres on linkage maps were delimited by FISH of marker-containing BACs. BACs from sorghum euchromatin typically yield readily identifiable locus-specific FISH signals. Collective results from successive sampling of landed BACs enabled the centromeres to be delimited on linkage maps. The chromosome-2 centromere was localized to between LG–B DNA markers *Xtxa6231* and *Xtxa363*, i.e., positions between 68.3 - 71.0 cM and 73.3 - 75.7 cM. Thus, the short arm of chromosome 2 encompasses DNA markers from map positions 0.0 to 71.0 cM, whereas the long arm encompasses map positions 73.3 to 205.2 cM. The centromere occurs in a large heterochromatic region that spans ~25% of chromosome 2, but is confined to a segment of the linkage map that is very short and accounts for only about ~2.4 % of its meiotic recombination.

FISH of landed BACs delimited the centromere of linkage group H to the segment between *Xtxa325* (69.8 cM) and *Xtxa2654 (*75.9 cM). DNA markers from map positions

0.0 to 69.8 cM were found to reside in the long arm and DNA markers from map positions 75.9 to 152.3 cM were located in the short arm of chromosome 8. The centromere is situated in a large heterochromatic region that spans ~50% of chromosome 8, but integration with the linkage map revealed that it accounts for merely 6.1% of its meiotic recombination. In chromosomes 2 and 8, pericentromeric regions are composed of a large block of heterochromatin. Disproportionately low rates of recombination in the pericentromeric regions have been reported in sorghum chromosome 1 (Islam-Faridi et al. 2002) as well as wheat, barley, and tomato (Tanksley et al. 1992; Delaney et al. 1995a, 1995b; Sherman and Stack 1995; Künzel et al. 2000).

FISH resolved the relative order of several DNA markers that had previously not been resolved by segregation analysis of linkage group B (*Xtxp84* and *Xtxp50*; *Xtxa6252* and *Xtxa3424*). FISH also resolved the relative order of two DNA markers that were co-localized in LG-H (*Xtxs2065* and *Xtxa388*). The physical resolution of linkage maps is subject to regional variation in the level of recombination. In physical regions that are low in recombination, the exact order of DNA markers and hence associated BAC clones cannot be easily resolved without complementary information. This study indicates that cytological analysis of somatic and/or pachytene sorghum chromosomes can eliminate ambiguity in at least certain regions of the sorghum linkage map.

BAC clones were evaluated by Southern hybridization and single-BAC FISH to determine the correlation between signal patterns after Southern hybridization with genomic DNA versus *in situ* hybridization of BAC probe without competition from C_0t-1 DNA. Most BAC libraries are comprised of genomic clones that are 100 - 200 Kb in

size, so the targets of BAC FISH are relatively large and easy to detect. Such large genomic clones, however, are more likely than small clones to contain dispersed repetitive sequences that cause high background signal after FISH (Hanson et al. 1995). With the aim of simultaneously localizing many BACs on individual sorghum chromosome spreads, we deemed it especially important to pick BACs with relatively low repetitive sequence content, and relatively high gene content or at least high unique sequence content. Detailed integration of maps across the entire genome will require the identification many BACs that are amenable to multi-probe FISH. Evaluation of each by single-probe FISH would be very time-consuming, so overall efficacy can be enhanced by development of facile non-FISH methods that enable selection BACs likely to yield locus-specific FISH signal and/or enhance their evaluation prior to FISH-based testing. Sorghum BACs that yielded the strongest signal on the Southern hybridization also yielded strongest background signals after FISH. BACs that yielded only moderate signal after the Southern hybridization with genomic DNA yielded good clean signal after FISH. Since numerous BACs can be tested per membrane, Southern hybridization enabled a facile means to large numbers of sorghum BAC clones likely to be amenable to FISH.

There are several possible approaches to physical map development through FISH of multiple probes. Among them are single-, dual- and multi-probe FISH. In some instances, a single slide has been used for multiple rounds of FISH, each with a different probe or small set of probes (Cheng et al. 2001). The purposeful selection of BACs for multi-probe FISH was pioneered in sorghum by Kim et al. (2002) and Islam-Faridi et al.

(2002). This approach enables greater efficacy, as a single FISH run requires less time to conduct, is simpler to interpret. Also, repeated FISH of a slide reduces the quality of preparations each round. On the other hand, simultaneous FISH of multi-BAC cocktails can lead to confluence FISH signals. The problem is greatest at mitotic metaphase, when chromosomes are shortest and neighboring signals thus closest. Although pachytene bivalents are much longer, the problem of signal confluence rises as the number of BACs in a chromosome-specific cocktail increases. Strategies to address this problem will be needed as the number and thus physical density of FISH signals rises. Our results with "overlapping" probe cocktails demonstrates that the inclusion of common BACs in "overlapping" probe cocktails enable facile multi-point alignment across chromosome (bivalent) spreads. Thus, any number of BACs can be used to create a common map.

CHAPTER V

ESTIMATING PHYSICAL DISTANCES AROUND TWO IMPORTANT GENES

OF SORGHUM FOR MAP-BASED CLONING: *ma$_5$* and *rf1*

Introduction

Sorghum (*Sorghum bicolor* Moench.) has perfect flowers and is naturally autogamous. The sorghum industry, however, relies completely on hybrid varieties, seed of which must be mass-produced using cytoplasmic male sterility and restoration (Schertz et al. 1989). The identification and development of male-sterile and fertility-restoration lines is essential for hybrid seed production in sorghum (Schertz and Dalton 1980). Once available, good molecular markers will facilitate the selection of pollen fertility restoration genes during sorghum inbred-line development. They could also provide the foundation for map-facilitated gene isolation. The mapping and tagging of the *rf1* locus in sorghum by amplified fragment length polymorphism (AFLP) and microsatellite simple sequence repeat (SSR) genetic markers have been reported (Klein et al., 2001). In the regional linkage map established around the *rf1* locus, *rf1* mapped to a position 2.4 cM from AFLP marker *Xtxa 2582*, and thus that the *rf1* locus maps to linkage group H of the high-density genetic map of sorghum (Klein et al., 2001). We have identified BAC clones containing the genetic markers flanking the *rf1* locus and used them for fluorescent *in situ* hybridization (FISH).

Sorghum is grown worldwide in an array of environments, ranging from tropical to

temperate. This array of environments has resulted in a range of maturities with differing degrees of photoperiod sensitivity. An understanding of the effect of day length on reproductive development has agronomic importance because the ability to alter flowering time allows the cultivation of a species in environments that differ greatly from the one in which it originally evolved. Photoperiod sensitivity of sorghum is controlled by at least six genes: Ma_1, Ma_2, Ma_3, Ma_4, Ma_5, and Ma_6 (Quinby 1967). The first four maturity genes cause long days to inhibit flowering but allow early flowering under short days. The genes Ma_5 and Ma_6 constitute a special case, because only when they are both present in the dominant form will they very strongly inhibit floral initiation regardless of day length. Isolating the Ma genes will help understand better their roles in photoperiod sensing in sorghum.

Genetic maps, constructed on the basis of recombination frequencies, are important tools in plant genetics (Davis et al. 1999). However, discrepancies have been demonstrated between genetic and physical maps, with variations between chromosomal regions (Künzel et al. 2000). Therefore, plant geneticists, instead of relying only on a linkage map, would find it highly advantageous to concomitantly use physical maps for delimiting, identifying and isolating genes. To establish the correlation between the physical and genetic maps also is essential for performing efficient map-based gene cloning and association candidate genes with important biological or agronomic traits. With respect to the isolation of genes, the feasibility of chromosome walking will be highly affected by the Mb/cM ratio (Civardi et al. 1994) and the prevalence of repetitive DNA sequences (Putterill et al. 1993).

In this study, the physical locations of molecular markers flanking two gene loci (*ma₅* and *rf1*) were determined on sorghum chromosomes by applying FISH. For construction of high-resolution physical maps using FISH, maximally extended pachytene bivalents were used since sorghum chromosomes isolated at the pachytene stage from pollen mother cells were about 10-fold longer than those from the mitotic metaphase stage.

Materials and Methods

Selection of BACs for FISH

The BACs used in this study came from two genomic BAC libraries that derived from sorghum cultivar BTx623 (Tao and Zhang 1998; Woo et al. 1994). BACs (sbb23575, sbb14482, sbb18071, sbb19883 and sbb12329) were selected using markers that collectively span the *rf1* locus (*Xtxp105, Xtxa606, Xtxp18, Xtxs2065 and Xtxa388*), and used to estimate physical distances along pachytene bivalents. For the estimation of physical distance around *ma₅* locus, BACs (sbb3687, sbb11773, sbb4217 and sbb10005) were selected with by markers (*Xtxa3803, Xtxa3424, Xtxp100* and *Xtxs1845*), which collectively define a segment that spans the *ma₅* locus.

Pachytene chromosome preparation

Pachytene chromosome preparation was performed according to the protocol of

Zhong et al. (1996). Young anthers from sorghum (*Sorghum bicolor* [L.] Moench.) plants (BTx623) were selected on the basis of stage for meiotic FISH preparation. Anthers at pachytene were rinsed in de-ionized water and then incubated at 37°C for 2 hr in enzyme solution (0.3% cellulase, 0.3% pectolyase and 0.3 cytohelicase in 10 mM citrate buffer, pH 4.5), rinsed with distilled water, placed on a clean glass slide with a drop of ethanol-acetic acid (3:1) fixative, macerated using fine-pointed forceps, allowed to air dry at RT for two days, and stored in a −20°C freezer.

BAC DNA purification and probe labeling

BAC DNA was isolated by alkaline lysis, digested with *Eco*RI, and then further purified using Plant DNeasy spin columns (Qiagen, Valencia, CA) using a modified protocol (Childs et al. 2001). The purified BAC DNA was labeled with biotin 16-dUTP or digoxigenin-11-dUTP by the BioNick Labeling system (Roche Molecular Biochemicals, Indianapolis, Indiana, USA).

***In situ* hybridization**

In situ hybridization techniques were a modification of Jewell and Islam-Faridi (1994) as described by Hanson et al. (1996).

Microscopy

Images were viewed through an Olympus AX-70 epifluorescence microscope equipped with standard filter cubes. Images from a Peltier-cooled 1.3 M pixel Sensys

camera (Roper Scientific) were captured with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, California, USA.).

<div align="center">

Results

</div>

Estimation of physical distance of *ma₅* locus

It was determined that the *ma₅* locus mapped to linkage group B of the high-density genetic map of sorghum. We have identified BAC clones containing the genetic markers flanking the *ma₅* locus and used them for fluorescent *in situ* hybridization (FISH). Selected BACs (sbb11773, sbb4217) using markers *Xtxa3624* and *Xtxp100* flanking to the *ma₅* locus were positioned in the middle of the euchromatic region of the long arm of chromosome 2 (Fig. 15). The physical distance of BACs (sbb11773, sbb4217) was estimated in Mb. Sorghum genome is 750 Mb. Considering that the relative length of chromosome 2 is 11.1% of the whole chromosomes of mitotic metaphase, chromosome 2 was estimated to have 83.25 Mb. Since the relative length between BAC sbb11773 and sbb4217 was 4.69% of pachytene bivalents of chromosome B, the length of genome calculated with the assumption of constant DNA density along chromosomes was 83.25 Mb $\times$ 4.69%= 3.90 Mb. The value of 3.90 Mb was overestimated because this region was an euchromatic region. However, though this region is considered as overestimated euchromatic region, still it is the distance where we need to develop more marker to reduce physical distance so that it can be a manageable distance for positioning cloning

(In the case of tomato, the genome size in the heterochromatic region was estimated 6 times than in the euchromatic region within the same length (Peterson et al.1996).

Estimation of physical distance around *rf1* locus on pachytene bivalents

It was determined that the *rf1* locus mapped to linkage group H of the high-density linkage map of sorghum. We have identified BAC clones containing the genetic markers flanking the *rf1* locus and used them for fluorescent *in situ* hybridization (FISH). Selected BACs (sbb18071 and sbb14482) using markers *Xtxp18* and *Xtxa60*6 flanking to the *rf*1 locus were positioned close to each other in the middle of the euchromatic region of the long arm of chromosome 8 (Fig. 16). Relative length of chromosome 8 is 8.8% of the whole chromosome of mitotic metaphase. Considering the genome size of sorghum as 750 Mb, chromosome 8 was estimated to have 66 Mb. Length of between sbb18071 and sbb14482 was estimated as 5.8% of pachytene chromosome 8. The calculated value, assuming a constant DNA density along chromosomes, was 66 Mb × 0.58 = 0.387 Mb. Since this region is euchromatin, the value of 0.387 Mb is overestimated. So, the real distance of this area should be smaller than that value. This information will aid the strategic use of BAC contigs to span the *rf1* locus and provide a starting point for identifying the responsible gene(s).

Fig. 15. Estimation of physical distances around *ma5* using pachytene FISH. (A) *In situ* hybridization to sorghum pachytene bivalents of BAC clones containing linkage markers that flank the *ma5* locus. (B) Partial linkage map of the segment of sorghum LG-B around the *ma5* locus.

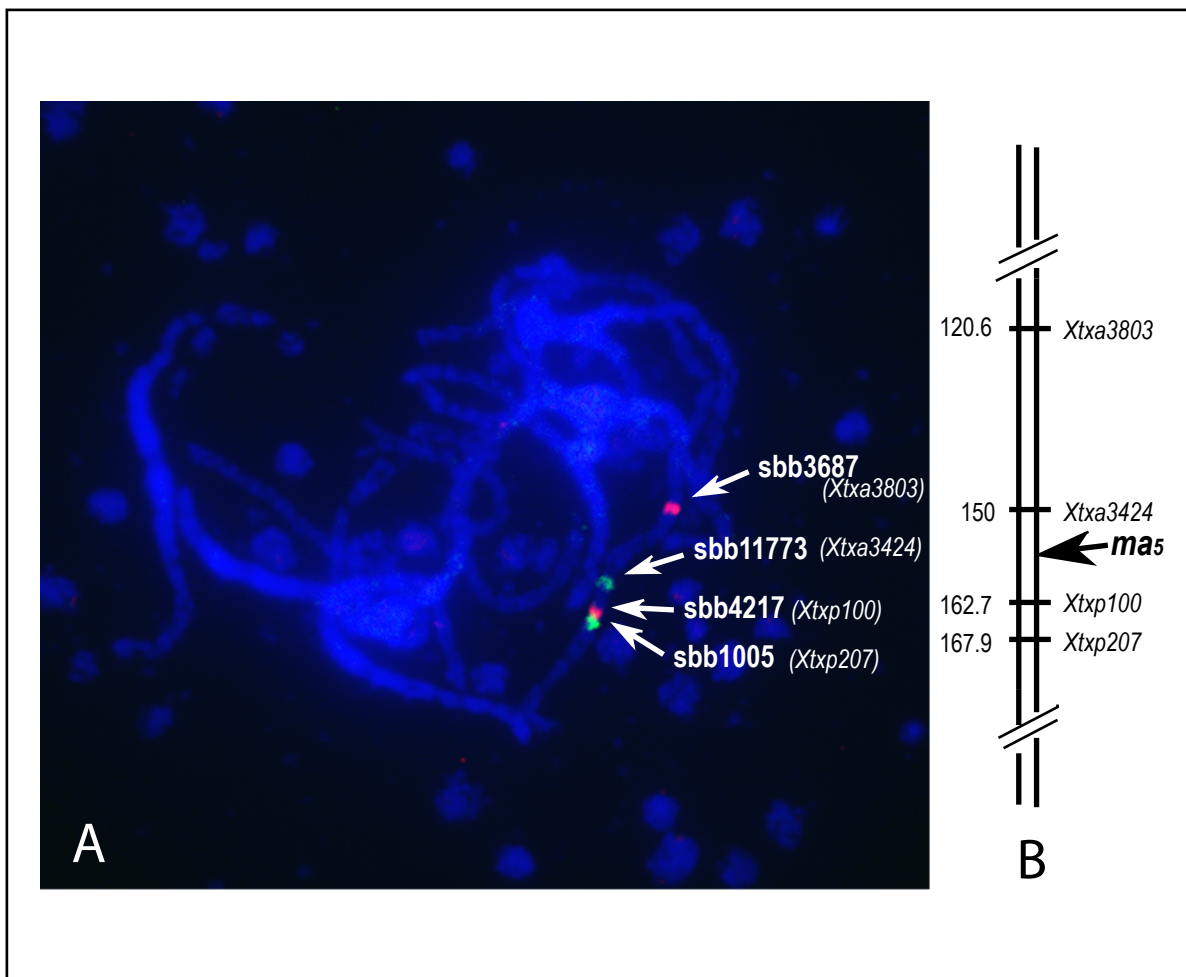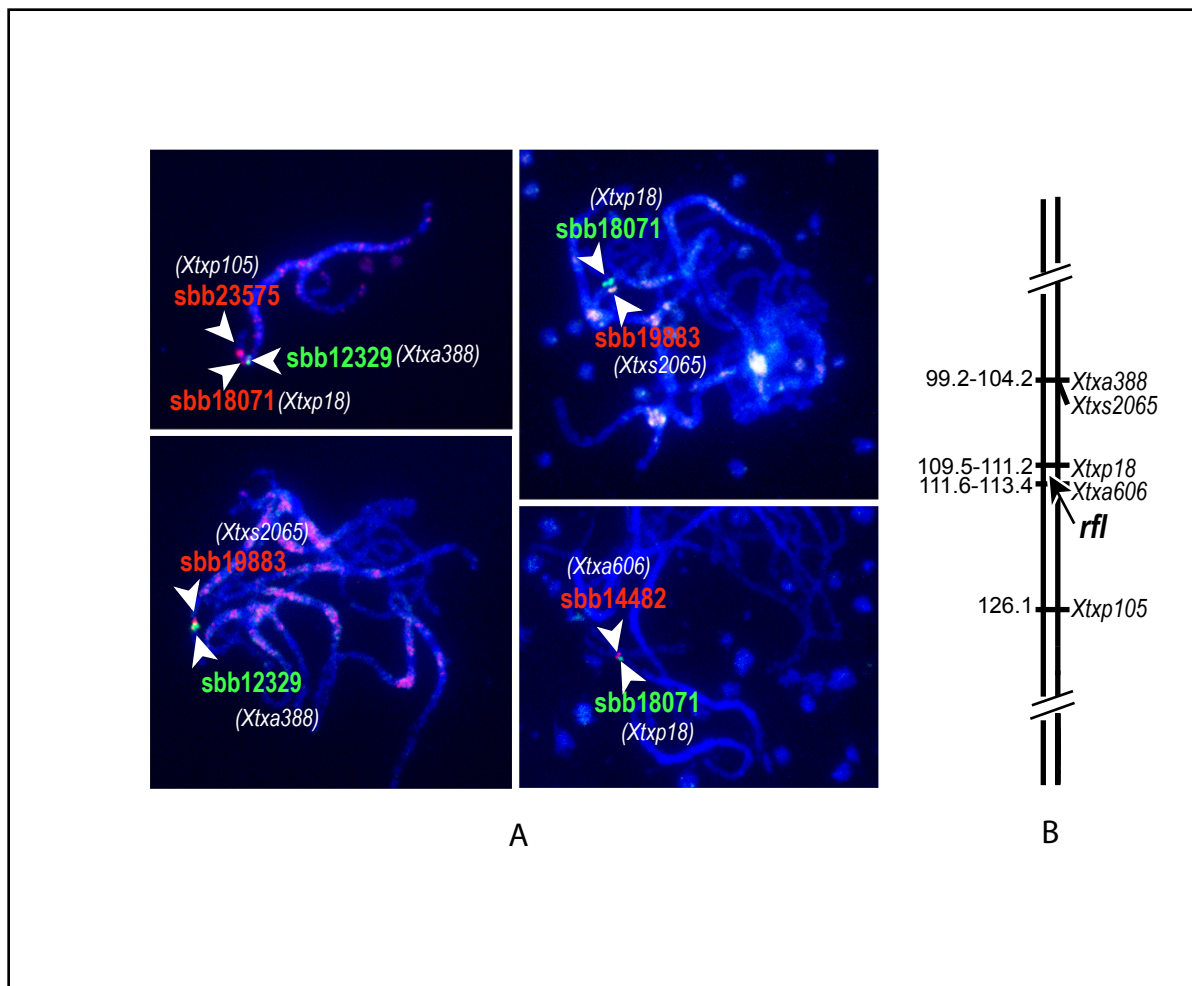**Fig. 16**. Estimation of physical distances around *rf1* using the pachytene FISH. (A) *In situ* hybridization to sorghum pachytene bivalents of BAC clones containing linkage markers that flank the *rf1* locus. (B) Partial linkage map of the segment of sorghum LG-H around the *rf1* locus.

Discussion

Cytogenetic or chromosome maps serve as intermediates between physical and genetic maps in displaying the approximate positions of genes and molecular markers, relative to structural markers, such as centromeres, telomeres, heterochromatic bands and secondary constrictions. They are unique in bringing together information from linkage maps and the distribution of crossover-events and physical (DNA) maps. Meiotic prophase chromosomes of plant species, in particular at pachytene, exhibit specific features, which make them particularly attractive for FISH studies. The sorghum chromosomes at meiotic prophase stage are 10 times longer than their counterparts at mitotic metaphase and allow mapping with a high resolution.

Based on the measurements of relative length of each chromosome and distance between the BAC signals around *rf1* locus, the size of *rf1* locus was estimated as 387 kb. This was assuming the constant DNA density along chromosomes, which caused overestimation since *rf1* was located in euchromatic region. In tomato, pachytene FISH can resolve probes separated by 1.2 Mb in heterochromatic regions and 120 kb in euchromatic regions (de Jong et al. 1999) and the resolution in euchromatic and heterochromatic regions in A. *thaliana* is 60 and 140 kb, respectively (de Jong et al. 1999). Considering those values, <387 kb might be reasonable estimation and distance between those two BACs, which was resolving distance on the euchromatin of pachytene chromosome (BAC clones separated by ~100kb can be well resolved on rice pachytene chromosomes (Cheng et al. 2001)). The indication that the *rf1* gene is in a

region of euchromatin within the area of relatively high recombination makes it a realistic target for cloning using a map-based strategy. We are currently generating further recombinants and anchoring BACs in this region for this purpose. Once isolated, the *rf1* gene will serve as an important tool for elucidating the developmental regulation of pollen fertility restoration gene.

In the case of *ma5*, the estimated physical distance of the region was <4 Mb, which also was overestimated because this region was euchromatic region. However, though considering this region as overestimated euchromatic region, still it is a long distance, compared with that in the case of the *rfl* locus. We might need to develop more markers to reduce physical distance so that it can be manageable distance for positioning cloning.

Clearly, FISH mapping of specific marker to meiotic pachytene chromosomes adds an extra dimension to the molecular genetic linkage maps of sorghum and should be regarded as being crucial before embarking on the positional cloning of a target gene merely on the basis of its very tight linkage to molecular markers. Moreover, the integrated cytogenetic-molecular map permits the estimation of distances up to mega base pairs between markers along the chromosome.

CHAPTER VI

DELIMITING CENTROMERES AND BOUNDARIES BETWEEN

EUCHROMATIN AND HETEROCHROMATIN IN SORGHUM

Introduction

The basic structure of eukaryotic chromosomes has long been viewed as being composed of two types of chromatin; euchromatin and heterochromatin. The term heterochromatin was originally used by Heitz (1929) who described a portion of the nuclear chromatin which maintains a condense state throughout cell interphase. Although heterochromatin is difficult to define, a number of properties have been described (Karpen and Allshire 1997), including suppression of both gene activity and genetic recombination. In sorghum, the majority of heterochromatin in each chromosome occurs as a "block" of pericentromeric heterochromatin. At certain stages, these pericentromeric regions correspond to intensely stained DAPI-positive regions. At pachytene, the bivalents display clear differences between the large heterochromatic blocks around the centromere and the euchromatic distal regions. At metaphase, however, the degree to which they are differentially stainable is minimal. This is when relative sizes of euchromatin and heterochromatin are most comparable, because, both are maximally contracted.

We estimated euchromatin and heterochromatin on the pachytene bivalent, where we can provide a higher resolution. To understand the organization of euchromatic and

heterochromatic components of an eukaryotic chromosome, it is necessary to relate the morphological features of chromosomes with genetic and molecular sequence data. The resulting integrated map will provide tools and information that enable correlations to be established between DNA sequences, structure, and function of the chromosome. It will aid efforts to determine the mechanism behind chromatin condensation, recombination, suppression and gene silencing of heterochromatin. It will also allow analysis of the overall architecture of the genome, including the size and distribution of the gene islands, the gene densities within these, and the range of gene structures.

Specific chromosomal regions have their own density of condensation pattern (Fukui and Mukai 1988). To reveal this characteristics in sorghum, we utilized digital image analysis to compare overall luminance profiles of pachytene bivalents with the longitudinal distribution of recombinational hot-spots and euchromatin versus heterochromatin along the individual chromosomal entities. The borders of condensed and dispersed regions within each chromosome were also distinguished by digital image analysis.

Materials and Methods

Preparation of metaphase chromosome

The degree to which chromosomes are differentially stainable is minimal at metaphase. This is when relative sizes of euchromatin and heterochromatin are most comparable, because, both are maximally contracted. Sorghum (*Sorghum bicolor* [L.] Moench.) plants (BTx623) were grown under glasshouse conditions. For collecting active metaphase chromosome, roots of BTx623 plant were cut and repotted and two days later, new root tips were collected. Saturated aqueous α-monobromonaphthalene was treated 2 hr, which is longer than usual so that chromosome was contracted without euchromatic tail left for maximum contraction.

Preparation of pachytene chromosome

The preparation of pachytene chromosome was performed according to the protocol of Zhong et al. (1996). Young anthers from Sorghum (*Sorghum bicolor* [L.] Moench.) plants (BTx623) were selected for the preparation of meiotic chromosome and fixed in ethanol-acetic acid (3:1) fixative. The stage of development was determined routinely in an aceto-carmine squash preparation using a single anther from a flower bud. If at prophase I, the remaining anthers were rinsed in de-ionized water and then incubated at 37°C for 2 hr in enzyme solution (0.3% cellulase, 0.3% pectolyase and 0.3 cytohelicase in 10 mM citrate buffer, pH 4.5), rinsed with distilled water, placed on a clean glass slide with a drop of ethanol-acetic acid (3:1) fixative, macerated using fine-pointed forceps,

allowed to air dry at RT for two days, and stored in a –20°C freezer.

Selection of BACs located on the border of euchromatin and heterochromatin using clustered markers on the genetic map

Sorghum has a big pericentromeric block in which is a suppressed genetic recombination. Centromeric regions are associated with the clustering of genetically unresolved markers on the molecular genetic linkage map (Tanksley et al. 1992). From these areas, genetic markers were selected, and using these markers, BACs were selected and tested for their locations on the pachytene bivalent by FISH. BACs, closest to the centromere from each side of arm, were selected and used for FISH on mitotic chromosomes.

Determination of the density in euchromatin versus heterochromatin

Unlike mitotic metaphase chromosomes, DNA density and chromosome diameter differ considerably between euchromatic and heterochromatic areas of pachytene chromosomes. After FISH, spreads of pachytene bivalents were stained with DAPI. Images were captured using Olympus AX-70 epifluorescence microscope, Peltier-cooled 1.3 M pixel Sensys camera (Roper Scientific) with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, Calif., U.S.A.). For estimating heterochromatin versus euchromatin, DAPI images were changed to gray images and inverted for better contrast before measurement using MacProbe. To assess relative strengths of FISH signals and their distributions, blue (4',6-diaminosino-2-2-

phenylindole [DAPI] signal from chromosomal DNA), green (FITC from probe) and red (Cy3 signal from probe) signals were measured from digital images using Optimas v6.0. Lines for sampling luminance values spanned the lengths of somatic chromosomes or meiotic bivalents. Data were extracted for the Optimas "linear morphology default data collection set," exported to a spreadsheet (Microsoft Excel).

Determination of the lengths of pachytene euchromatin versus heterochromatin

To estimate relative amounts of euchromatin and heterochromatin on a per chromosome basis and overall, we used mitotic metaphase FISH of BACs that we had previously determined by pachytene FISH to be close to the border of euchromatin and heterochromatin. Mitotic metaphase chromatin is maximally contracted, so the relative densities of euchromatin and heterochromatin are most equal at that stage. For purposes of estimation, densities of euchromatin and heterochromatin were assumed to be equal at mitotic metaphase.

Results

Selection of BACs located on the border of euchromatin and heterochromatin

Centromeric regions are associated with the clustering of markers unresolved on the molecular genetic linkage map (Tanksley et al. 1992). Markers from these regions (centromeric region of genetic map) corresponded to BACs around the border of euchromatin and heterochromatin region. Screened BACs, anchored by markers from those regions, were checked for their locations on the pachytene bivalents (Fig. 17). BACs, located on the border of euchromatin and heterochromatic region were selected for estimation of the length of euchromatin and heterochromatin region, and for the comparison of genetic and physical distance.

Estimation of arm ratio and euchromatin versus heterochromatin of the pachytene bivalents 2 and 8

Using the specific probe pCEN 38 probe, centromere was located on the pachytene bivalents. Pachytene bivalent 2 has an arm ratio of 1:1.94 and pachytene bivalent 8 has an arm ratio of 1:1.3. In both of cases, the orientation of short arm and long arm was same as that of their corresponding metaphase chromosomes. Based on the densitometric profiles, euchromatin and heterochromatin was distinguished (Fig. 18). Chromosome 2 has 25.78% of heterochromatin in relative length and almost same amount of heterochromatin was distributed on its short arm (12.11%) and its long arm (13.67%). 21.88% and 52.34% of relative length of euchromatin was distributed on its

short arm and on the long arm, respectively. Checking with the markers (*Xtxa6231 and Xtxa3634*) anchored BACs (sbb8236 and sbb1819) near this heterochromatin border, this physical distance (25.78%) corresponded to 2.85% (5.85cM / 205cM) on the genetic map.

In the case of chromosome 8, heterochromatin was 49.61% of the chromosome length. Euchromatin of short arm and long arm was 17.99% and 32.42%, respectively. Meanwhile, heterochromatin of short arm and long arm was almost same length (25.39% and 24.22%). *Xtxa209* anchored BAC (sbb4303), located on the border of euchromatin and heterochromatin of short arm, and *Xtxa3968* anchored BAC (sbb18981), located little further to euchromatin side of long arm. Calculated genetic distance (74.55 - 84.25 = 9.7 cM (6.37%)) corresponded to 49.61% of physical distance on the pachytene chromosomes.
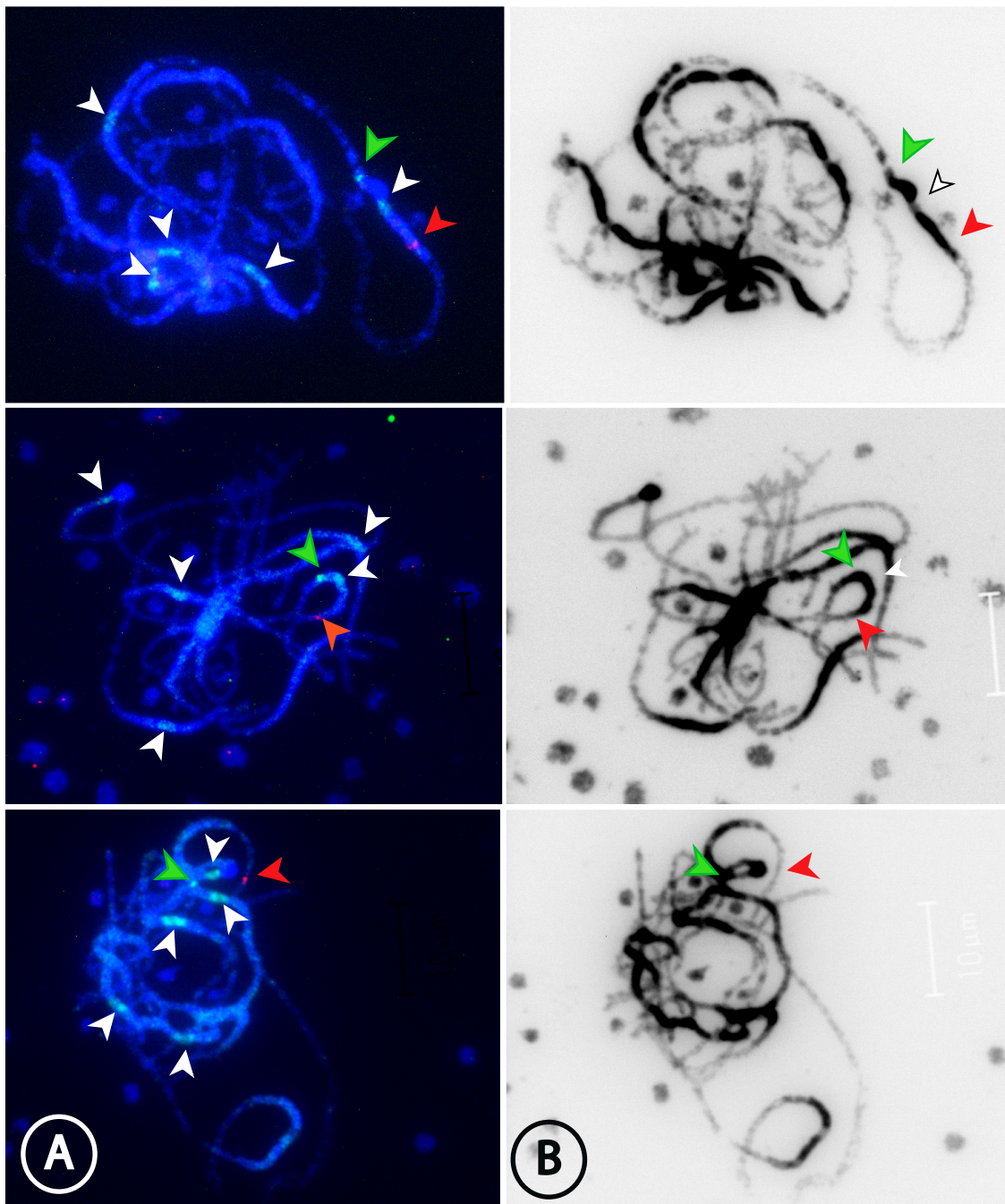
Fig. 17. Delimitation of border between euchromatin and pericentromeric heterochromatin. (A) FISH result of landed BACs checking the location of the BAC signal to delimit the borders between euchromatin and heterochromatin. White arrow heads indicate signals of pCEN38   located at the centromere and green and red arrow heads indicate BAC signals. (B) Inverted grayscale images of Fig. A for better visualization of heterochromatin versus

Fig. 18. Estimation of euchromatin versus heterochromatin using FISH for pachytene bivalents of *Sorghum bicolor*. (A) FISH image of two BACs (sbb8236 and sbb1819) located at the border of euchromatin and heterochromatin and pCEN 38 to identify centromeric regions. White arrowhead indicates pCEN38 signal and red (sbb8236 and sbb1819) and green (sbb10660) arrowheads indicate BAC signals. (B) DAPI image. (C) Inverted gray scale images showing better visualization of euchromatin versus heterochromatin. (D) Luminance profile showing relative strengths of fluorochrome and FISH signals; blue (4',6-diaminosino-2-2-phenylindole [DAPI] signal from chromosomal DNA), green (FITC from probe) and red (Cy3 signal from probe) signals.
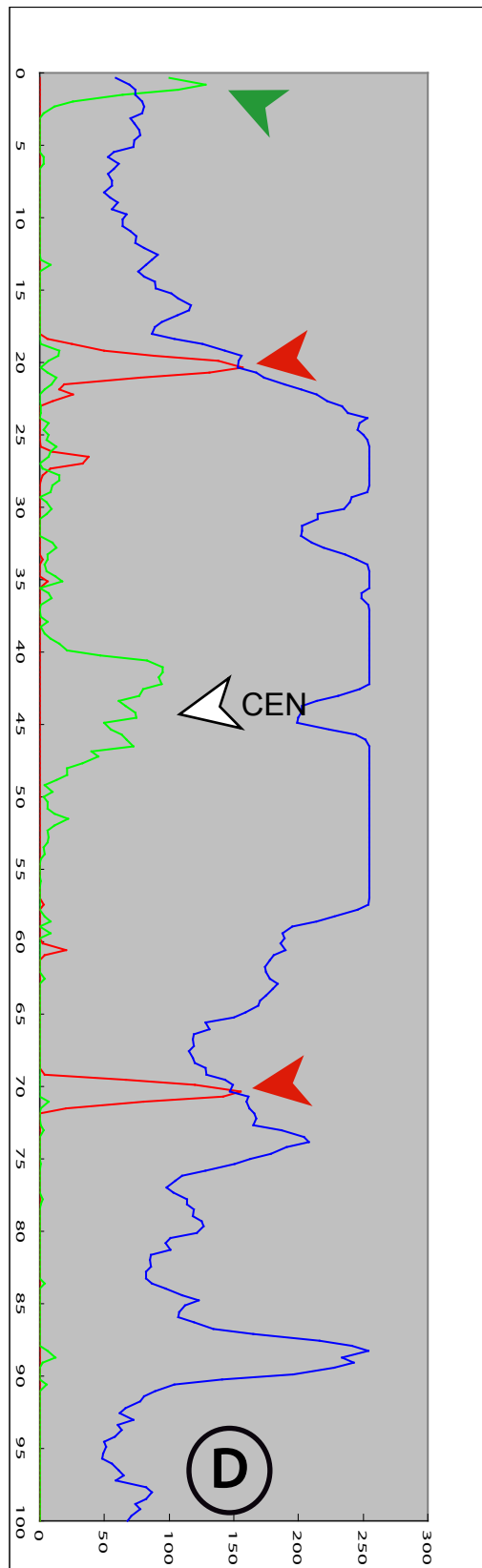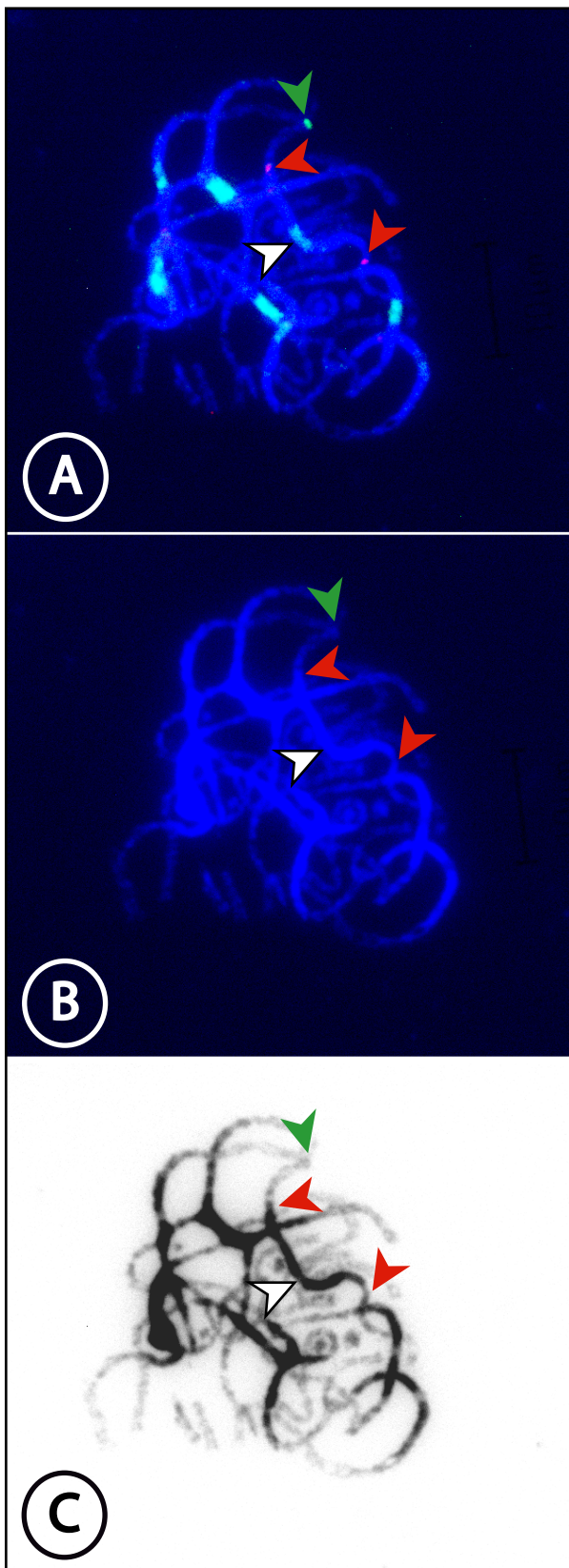
Centromere mapping

BACs located closest to centromere of each side of arm, were selected to map the centromere roughly on the genetic maps (Table 5 and Fig. 19). Centromeres were mapped in the genetic distance of 0 ~ 22.9%. Within 0 ~ 2-3%, centromeres were mapped in the linkage group A, B, and C, while their corresponding distance on the metaphase was around 25 - 20%. In the rest of linkage group, centromeres were mapped in the map distance of 5.9% - 19.78%, but all cases have their corresponding physical distance on the metaphase >50%.

Comparison of lengths of euchromatin and heterochromatin on the mitotic chromosome and pachytene bivalents (Figs. 18 and 19)

Two BACs, located on the border of euchromatin and heterochromatin, were selected to estimate the relative distance around the heterochromatin both on the mitotic and pachytene chromosomes. Relative length between two closest BACs around the pachytene chromosome 2 was 23.5%, and from the eye-view, the relative length on the mitotic metaphase chromosome was longer than that on the pachytene chromosome. On the pachytene chromosome 8, two closest BACs around centromere spanned 50% of relative distance, but in the mitotic chromosome, it spanned more than 50%. This is the reason for the higher ratio of contraction between euchromatin and heterochromatin in the mitotic metaphase than in the pachytene chromosome. Because of this reason, arm ratio was bigger in the case of pachytene chromosome.

Table 5. List of BAC clones used for FISH to map centromeres (Fig. 19), their associated linkage markers, and locations on linkage maps.

| Linkage group | Short arm | | | Long arm | | | Total map length |
	Clone (sbb)	Marker	Location (cM)	Clone (sbb)	marker	Location (cM)	cM
LG-A	3890	*Xtxa3292*	75.6-80.5	18256	*Xtxa325*	75.6-80.5	232.2
LG-B	8236	*Xtxa6231*	68.3-71.0	1819	*Xtxa3634*	73.3-77.7	205.2
LG-C	21451	*Xtxa2904*	76.5-79.9	16961	*Xtxa3618*	76.5-79.9	196.5
LG-D	22928	*Xtxa3955*	86.1	10455	*Xtxa2235*	100.7-110.6	174.6
LG-E	8215	*Xtxa307*	73.9	2754	*Xtxa6333*	85.9-88.0	155.9
LG-F	10168	*Xtxa3765*	72.8-81.6	16550	*Xtxa4113*	85	153
LG-G	24082	*Xtxa3607*	64.3	10164	*Xtxa4172*	77.9-80.3	148
LG-H	4303	*Xtxa209*	72.7-76.4	18981	*Xtxa3968*	82.5-86.0	152.3
LG-I	10183	*Xtxp6*	0	25146	*Xtxa4103*	22.9	115.8
LG-J	19655	*Xtxa2038*	57.6	7239	*Xtxa4081*	80.3	118
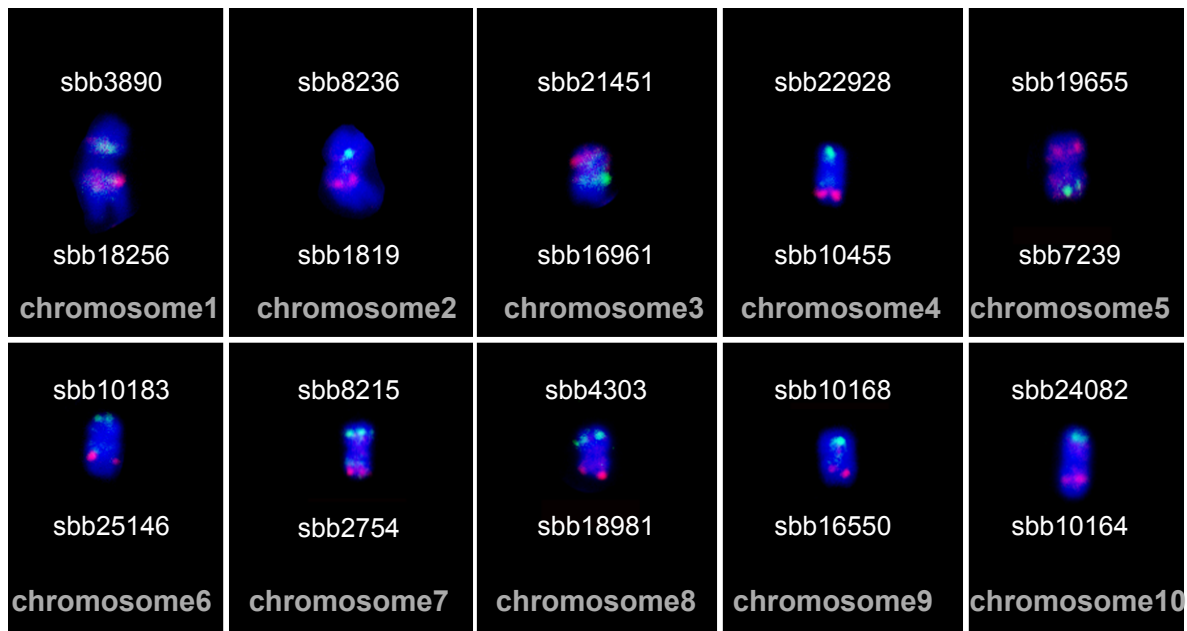
Fig. 19. FISH results for the two BACs closest to each side of centromere for all ten chromosomes. BAC names located on the upper side of each chromosome correspond to the signal on the short arm and BAC names located on the lower side of each chromosome correspond to the signal on the long arm.

Discussion

Clustering of AFLP markers has been reported in numerous genetic maps (Powell et al. 1997; Alonso-Blanco et al. 1998; Qi et al. 1998; Boivin et al. 1999). It has often been assumed that these clusters correspond to regions of the genome which are hypermethylated and have relatively low rates of recombination, such as centromeres. Menz et al. (2002) observed some aggregation of the EcoRI/MseI markers in sorghum linkage maps, but it was unclear whether such clustering occurs around centromeres and/or other regions. Using markers from these marker-rich regions, we found by FISH of landed BACs that the regions were in centromeric regions. By delimiting borders of the pachytene bivalent pericentromeric heterochromatin and assignment of markers to arms of mitotic chromosomes, locations of centromeres on the 10 sorghum linkage maps were roughly estimated. For BACs that FISH localized to centromere regions, all of the BAC-contained linkage markers were clustered within a very short distance on the linkage map. In sorghum, short distances along the genetic map around centromere correspond to huge blocks of pericentric heterochromatin.

The Gramineae vary 5-fold in ploidy level, 30-fold in chromosome number and 100-fold in DNA content (Bennett and Leitch 2003). The large variation in genome size observed in the grass family is thus attributable only in part to differences in ploidy. Much of the variation arose from repetitive elements, primarily retrotransposons, located between low copy number genic regions in the genome (San Miguel et al. 1996). In maize (*Zea mays*), retrotransposons are estimated to make up 50% to 80% of the genome (San Miguel and Bennetzen 1998). Gene density values determined by direct sequencing

around orthologous loci in grass species with small and large genomes vary only 2- to 5-fold, a value relatively small considering the disparate sizes of grass genomes (Feuillet and Keller 1999; Tikhonov et al. 1999; Tarchini et al. 2000). Morishige et al. (2002) further substantiated the notion that genes are concentrated in particular regions of the sorghum genome with other regions being relatively gene poor.

Identification of euchromatin and heterochromatin at both the cytological and DNA sequence level will be a critical component for full characterization of the plant genome. The power and importance of integration of molecular and cytological data was distinctly illustrated by the recent characterization of a heterochromatic structure on the short arm of *Arabidopsis thaliana* chromosome 4 (Fransz et al. 2000; McCombie et al. 2000.) and rice (Cheng et al. 2001). DAPI-bright regions cover significant parts of the pericentric regions in the majority of the sorghum chromosomes. FISH-based analyses will be critical to integrate DNA sequence information with different types of chromatin structures. Determining the distribution of heterochromatin and euchromatin and the frequency of genetic recombination within these regions is important to establish a guide to future genome sequencing activities and map-based gene cloning.

Pachytene bivalent luminescence profiles revealed the longitudinal distribution of heterochromatin versus euchromatin bands along the individual chromosomes. At pachytene, the pericentromeric regions were intensely stained DAPI-positive regions. From the spatial representation of the chromosome images, it is evident that standard DAPI staining generates discernible density characteristics of the defined chromosomal domains. The corresponding densitometric profiles reflected the density distribution

along the chromosome. They provided a readily tabulated and visualized account of the general condensation patterns and local fluctuation in chromatin compactness. It is important to mention that the borders of condensed and dispersed regions within each chromosome were easily distinguishable on graphic plots of the density distributions.

In sorghum, the majority of heterochromatin in each chromosome occurs as a "block" of pericentromeric heterochromatin. DAPI-bright regions of the genome are denoted as heterochromatic ones and the observed correlations make digital analysis an adequate tool for screening of both inert and active genomic regions. The validity of this statement was supported also from the fact that recombinational hot-spots were found to reside in chromosomal segments with low density characteristics, i.e., the euchromatic ones.

Metric data on the chromosomes, chromatin features, FISH of region-specific BACs and the centromere region-specific clone pCEN38 enabled arm ratio estimate for pachytene bivalents and mitotic chromosomes. The arm ratios from pachytene bivalents were higher than the arm ratios from mitotic chromosomes. Differences in contraction are seen between euchromatin and heterochromatin, but seem to be minimized at metaphase, when both are maximally contracted. Euchromatin is far more contracted in mitotic chromosomes than at pachytene. So, estimates taken of relative sizes (Mb) of euchromatic and heterochromatic segments might be most directly comparable. Upon acquiring more data on signal locations and chromosome features, it will be possible to more reliably estimate arm ratios for both chromosomes, and interpret effects of condensation on arm lengths and ratios across different cell types and stages.

CHAPTER VII

CONCLUSIONS

The development of reliable and simple techniques for chromosome identification is critical for genome analysis and cytogenetics. Unfortunately, chromosome identification is a major challenge in many species with small chromosomes. The difficulty of identifying small chromosomes and segments tends to be exacerbated in plants, for most which high-resolution banding technologies are still lacking. In this work, the lack of high-resolution banding techniques was side-stepped by using fluorescent *in situ* hybridization (FISH) in concert with integrated structural genomic resources, including high-resolution linkage maps and large-insert genomic clones in bacterial artificial chromosome (BAC) libraries. In this experiment, FISH signals allowed chromosome pairs to be identified easily and unambiguously. The results enabled integration of the genetic map at the cytological level with specific chromosomes, and the orientation of individual linkage groups with respect to chromosome ends.

While the order of loci between linkage and physical maps was highly concordant, strong discrepancies existed between relative distances between loci at the recombinational and physical levels. When working at the molecular level, plant geneticists will avoid many pitfalls by not relying exclusively on its genetic map, as many inferences drawn therefrom will be grossly inaccurate. Rather, they would find it highly valuable use an integrated linkage-physical map for mapping and isolating genes.

In this study, linkage maps were used simultaneously to develop and integrate with

physical maps, by pachytene FISH of LG marker-anchored BACs. This delivered directly visible physical evidence of the order and physical positions of molecular markers on the chromosomes. The comparison of physical maps and linkage maps enabled us to reveal the gross relationship between physical distances and genetic distances for the entire sorghum genome. Extending the approach to specific regions, we used flanking markers to analyze the regions around of two important genes (*ma$_5$, rfl*). Such information is critical to assessing the feasibility of map-assisted cloning and selecting a suitable strategy. Generally speaking, the feasibility of "chromosome walking" will be highly affected by the Mb/cM ratio and the prevalence of repetitive DNA sequences, both of which can be readily assessed from *ad hoc* analysis or a comprehensive integrated map.

Sorghum chromosomes have big heterochromatin blocks around their respective pericentric regions, where the recombination rate is severely suppressed. This suggests that other (gene-rich) regions of sorghum may be reasonably close to rice in terms of their gene density in gene-rich regions. This inference is in keeping with sequence and functional genomic (EST) analysis of gene-rich BACs.

The tremendous success of these studies point clearly to the opportunity and need to expand on this "cyto-genomic" approach. Sorghum is an important crop, about which much is yet to be learned and gained. Integrated cyto-genomic resources will enhance many sorts of endeavor -- genomics, germplasm characterization, germplasm utilization, marker-assisted selection and cloning. Furthermore, sorghum's phylogenetic position indicates that understanding its genomics could be parlayed into a more insightful

understanding of the many complexities in plant genome evolution.   In summary, techniques for *in situ* hybridization to mitotic chromosome for karyotyping and to pachytene bivalents for developing physical mapping are very valuable for molecular cytogenetic studies. The integration of various types of maps enhances the reliability and robustness of individual maps, and extends their utility.

REFERENCES

Alonso-Blanco, C., Peeter, A. J. M., Koornneef, M., Lister, C., Dean, C., van den Bosch, N., Pot, J., and Kuiper, M. T. R. 1998. Development of an AFLP based linkage map of L*er*, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a Ler/Cvi recombinant inbred line population. Plant J. **14:** 259-271.

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796-815.

Armstrong, S.J., Fransz, P., Marshall, D.F., and Jones, G.H. 1998. Physical mapping of DNA repetitive sequences to mitotic and meiotic chromosomes of *Brassica oleracea* var. *alboglabra* by fluorescence *in situ* hybridization. Heredity **81:** 666-673.

Arumuganathan, K., and Earle, E.D. 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. **9:** 208-218.

Barakat, A., Carels, N., and Bernardi, G. 1997. The distribution of genes in the genomes of Gramineae. Proc. Natl. Acad. Sci. USA **94:** 6857-6861.

Barakat, A., Han, D.T., Benslimane, A.A., Rode, A., and Bernardi, G. 1999. The gene distribution in the genomes of pea, tomato and date palm. FEBS Letters **463:** 139-142.

Bennett MD, Leitch IJ. 2003. Angiosperm DNA C-values database (release 4.0, Jan. 2003) http://www.rbgkew.org.uk/cval/homepage.html

Bhattramakki, D., Dong, J., Chhabra, K., and Hart, G.E. 2000. An integrated SSR and RFLP linkage map of *Sorghum bicolor* (L.) Moench. Genome **43:** 988-1002.

Blanc, G., Barakat, A., Guyot, R., Cooke, R., and Delseny, I. 2000. Extensive duplication and reshuffling in the arabidopsis genome. Plant Cell **12:** 1093-1101.

Blum, S., Ramaiah, S., Kanemasu, E.T., and Paulsen, G.M. 1990. The physiology of heterosis in sorghum with respect to environmental stress. Ann. Bot. **65:** 149-158.

Boivin, K., Deu, M., Rami, J-F., Trouche, G., and Hamon, P. 1999. Towards a saturated sorghum map using RFLP and AFLP markers. Theor Appl. Genet. **98:** 320- 328.

Bonnema, G., Schipper, D., van Heusden, S., Zabel, P., and Lindhout, P. 1997. Tomato chromosome 1: high resolution genetic and physical mapping of the short arm in an interspecific *Lycopersicon esculentum × L. peruvianum* cross. Mol. Gen. Genet. **253:** 455-462.

Broman, K.W., Murray, J.C., Sheffield, V.C., White, R.L., and Weber, J.L. 1998. Comprehensive human genetic maps: individual and sex-specific variation in recombination. Am. J. Hum. Genet. **63:** 861-869.

Brown, G.R., and Carlson, J.E. 1997. Molecular cytogenetics of the genes encoding 18S-5.8S-26S rRNA and 5S rRNA in two species of spruce (*Picea*). Theor. Appl. Genet. **95:** 1-9.

Brown, G.R., Newton, C.H., and Carlson, J.E. 1998. Organization and distribution of a Sau3A tandem repeated DNA sequence in *Picea* (Pinaceae) species. Genome **41:** 560-565.

Chen, C.C., Chen, C.M., Hsu, F.C., Wang, C.J., Yang, J.T., and Kao, Y.Y. 2000. The pachytene chromosomes of maize as revealed by fluorescence *in situ* hybridization with repetitive DNA sequences. Theor. Appl. Genet. **101:** 30-36.

Cheng, Z., Presting, G.G., Buell, C.R., Wing, R.A., and Jiang, J. 2001. High-resolution pachytene chromosome mapping of bacterial artificial chromosomes anchored by genetic markers reveals the centromere location and the distribution of genetic recombination along chromosome 10 of rice. Genetics **157:** 1749-1757.

Cheung, V.G., Nowak, N., Jang, W., Kirsch, I.R., Zhao, S., Chen, X.-N., Furey, T.S., Kim, U.-J., Kuo, W.-L., Olivier, M., Conroy, J., Kasprazyk, A., Massa, H., Yonescu, R., Sait, S., Thoreen, C., Snjders, A., Lemyre, E., Bailey, J.A., Bruzel, A., Burrill, W.D., Clegg, S.M., Collins, S., Dhami, P., Friedman, C., Han, C.S., Herrick, S., Lee, J., Ligon, A.H., Lowry, S., Morley, M., Narasimhan, S., Osoegawa, K., Peng, Z., Plajzer-Frick, I., Quade, B.J., Scott, D., Sirotkin, K., Thorpe, A.A., Gray, J.W., Hudson, J., Pinkel, D., Ried, T., Rowen, L., Shen-Ong, G.L., Strausberg, R.L., Birney, E., Callen, D.F., Cheng, J.-F., Cox, D.R., Doggett, N.A., Carter, N.P., Eichler, E.E., Haussler, D., Korenberg, J.R., Morton, C.C., Albertson, D., Schuler, G., de Jong, P.J., and Trask, B.J. 2001. Integration of cytogenetic landmarks into the draft sequence of the human genome. Nature **409:** 953-958.

Childs, K.L., Klein, R.R., Klein, P.E., Morishige, D.T., and Mullet, J.E. 2001. Mapping genes on an integrated sorghum genetic and physical map using cDNA selection technology. Plant J. **27:** 243-255.

Chittenden, L.M., Schertz, K.F., Lin, Y., Wing, R., and Paterson, A.H. 1994. A detailed RFLP map of *Sorghum bicolor* X *S. propinquum*, suitable for high-density mapping, suggests ancestral duplication of *Sorghum* chromosomes or chromosomal segments. Theor. Appl. Genet. **87:** 925-933.

Civardi, L., Xia, Y., Edwards, K.J., Schnable, P.S., and Nikolau, B.J. 1994. The relationship between genetic and physical distances in the cloned *al-sh2* interval of the *Zea mays* L. genome. Proc. Natl. Acad. Sci. USA **91:** 8268-8272.

Collins, A., Frezal, J., Teague, J., and Morton, N.E. 1996. A metric map of humans: 23,500 loci in 850 bands. Proc. Natl. Acad. Sci. USA **93:** 14771-14775.

Crane, C.F., Price, H.J., Stelly, D.M., Czeschin, D.G., Jr., and McKnight, T.D. 1993. Identification of a homeologous chromosome pair by *in situ* DNA hybridization to ribosomal RNA loci in meiotic chromosomes of cotton (*Gossypium hirsutum*). Genome **36:** 1015-1022.

Crasta, O.R., Xu, W.W., Rosenouw, D.T., Mullet, J., and Nguyen, H.T. 1999. Mapping of post-flowering drought resistance traits in grain sorghum. Association between QTLs influencing premature senescence and maturity. Mol. Gen. Genet. **262:** 579-588.

Davis, L.G., McMullen, M.D., Baysdorfer, C., Musket, T., Grant, D., Staebell, M., Xu, G., Polacco, M., Koster, L., Melia-Hancock, S., Houchins, K., Chao, S., and Coe, E.H. Jr. 1999. A maize map standard with sequenced core markers, grass genome reference points and 932 expressed sequence tagged sites (ESTs) in a 1736-locus map. Genetics **152:** 1137-1172.

de Donato, M., Gallagher, D.S., Davis, S.K., Ji, Y., Burzlaff, J., Stelly, D.M., Womack, J.E., and Taylor, J.F. 1999. Physical assignment of microsatellite-containing BACs to bovine chromosomes. Cytogenet. Cell Genet. **87:** 59-61.

de Jong, J.H., Fransz, P., and Zable, P. 1999. High resolution FISH in plants -techniques and applications. Trends Plant Sci. **4:** 258-263.

Delaney, D., Nasuda, S., Endo, T.R., Gill, B.S., and Hulbert, S.H. 1995a. Cytogenetically based physical maps of the group-2 chromosomes of wheat. Theor. Appl. Genet. **91**: 568-573.

Delaney, D., Nasuda, S., Endo, T.R., Gill, B.S., and Hulbert, S.H. 1995b. Cytogenetically based physical maps of the group-3 chromosomes of wheat. Theor. Appl. Genet. **91**: 780-782.

Dhar, M.K., Kaul, S., Friebe, B., and Gill, B.S. 2002. Chromosome identification in *Plantago ovata* Forsk. through C-banding and FISH. Current Science **83:** 150-152.

Doggett, H. 1988. Sorghum. 2nd ed. John Wiley and Sons, Inc., New York.

Dong, F., Song, J., Naess, S.K., Helgeson, J.P., Gebhardt, C., and Jiang, J. 2000. Development and applications of a set of chromosome-specific cytogenetic DNA markers in potato. Theor. Appl. Genet. **101:** 1001-1007.

Endo, T.R., and Gill, B.S. 1984. The heterochromatin distribution and genome evolution in diploid species of *Elymus* and *Agropyron*. Can. J. Genet. Cytol. **26:** 669-678.

Endo, T.R., and Gill, B.S. 1996. The deletion stocks of common wheat. J. Hered. **87:** 295-307.

Faris, J.D., Haen, K.M., and Gill, B.S. 2000. Saturation mapping of a gene-rich recombination hot spot region in wheat. Genetics, **154:** 823-835.

Feuillet, C., and Keller, B. 1999. High gene density is conserved at syntenic loci of small and large grass genomes. Proc. Natl. Acad. Sci. USA **96:** 8265-8270.

Fillon, V., Morisson, M., Zoorob, R., Auffray, C., Douaire, M., Gellin, J., and Vignal, A. 1998. Identification of 16 chicken microchromosomes by molecular markers using

two-colour fluorescence *in situ* hybridization (FISH). Chromosome Research **6:** 307-313.

Fransz, P.F., Alonso-Blanco, C., Liharska, T.B., Peeters, A.J.M., and Zabel, P., and de Jong, J.H. 1996. High-resolution physical mapping in *Arabidopsis thaliana* and tomato by fluorescence *in situ* hybridization to extended DNA fibers. Plant J. **9:** 421-430.

Fransz, P.F., Armstrong, S., Alonso-Blanco, C., Fischer, T.C., Torres-Ruiz, R.A., and Jones, G.H. 1998. Cytogenetics for the model system *Arabidopsis thaliana*. Plant J. **13:** 867-876.

Fransz, P.F., Armstrong, S., de Jong, J.H., Parnell, L.D., van Drunen, C., Dean, C., Zabel, P., Bisseling, T., Jones, G.H. 2000. Integrated cytogenetic map of chromosome arm 4S of *A. thaliana*: structural organization of heterochromatic knob and centromere region. Cell **100:** 367-376.

Fuchs, J., Kühne, M., and Schubert, I. 1998. Assignment of linkage groups to pea chromosomes after karyotyping and gene mapping by fluorescence *in situ* hybridization. Chromosoma **107:** 272-276.

Fukui, K., and Mukai, Y. 1988. Condensation pattern as a new image parameter for identification of small chromosomes in plants. Jpn. J. Genet. **63:** 359-366.

Gall, J.G., and Pardue, M.L. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. Natl. Acad. Sci. USA **63:** 378-383.

Gerton, J.L., DeRisi, J., Shroff, R., Lichten, M., Brown, P.O., and Petes, T.D. 2000. Inaugural Article: global mapping of meiotic recombination hotspots and coldspots

in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **97:** 11383-11390.

Gill, K.S., Gill, B.S., and Endo, T.R. 1993. A chromosome region-specific mapping strategy reveals gene-rich telomeric ends in wheat. Chromosoma, **102:** 374-381.

Gill, K.S., Gill, B.S., Endo, T.R., and Taylor, T. 1996. Identification and high-density mapping of gene-rich regions in chromosome group 1 of wheat. Genetics **144:** 1883-1891.

Gingrich, J.C., Shadravan, F., and Lowry, S.R. 1993. A fluorescence *in situ* hybridization map of human chromosome 21 consisting of 30 genetic and physical markers on the chromosome: localization of 137 additional YAC and cosmid clones with respect to this map. Genomics **17:** 98-105.

Gomez, M.I. 1997. Molecular cytogenetic physical mapping of *Sorghum bicolor* chromosomes. Ph. D. dissertation. Texas A&M University, College Station.

Gomez, M.I., Islam-Faridi, M.N., Woo, S.S., Schertz, K.F., Czeschin, D. Jr., Zwick, M.S., Wing, R.A., Stelly, D.M., and Price, H.J. 1997. FISH of a maize *sh2*-selected sorghum BAC to chromosomes of *Sorghum bicolor*. Genome **40:** 475-478.

Gu, M.-H., Ma, H.-T., and Liang, G.H. 1984. Karyotype analysis of seven species in the genus *Sorghum*. J. Hered. **75:** 196-202.

Hanson, R.E., Zwick, M.S., Choi, S.D., Islam-Faridi, M.N., McKnight, T.D., Wing, R.A., Price, H.J., and Stelly, D.M. 1995. Fluorescent *in situ* hybridization of a bacterial artificial chromosome. Genome **38:** 646-651.

Hanson, R.E., Islam-Faridi, M.N., Percival, E.A., Crane, C.F., McKnight, T.D., Stelly, D.M., and Price, H.J. 1996. Distribution of 5S and 18S-28S rDNA loci in a tetraploid

cotton (*Gossypium hirsutum* L.) and its putative diploid ancestors. Chromosoma **105:** 55-61.

Hanson, R.E., Zhao, X.-P., Islam-Faridi, M.N., Paterson, A.H., Zwick, M.S., Crane, C.F., McKnight, T.D., Stelly, D.M., and Price, H.J. 1998. Evolution of interspersed repetitive elements in *Gossypium* (Malvaceae). Am. J. Bot. **85:** 1364-1368.

Harushima, Y., Yano, M., Shomura, A., Sato, M., Shimano, T., Kuboki, Y., Yamamoto, T., Lin, S.Y., Antonio, B.A., Parco, A., Kajiya, H., Huang, N., Yamamoto, K., Nagamura, Y., Kurata, N., Khush, G.S., and Sasaki, T. 1998. A high-density rice genetic linkage map with 2275 markers using a single F_2 population. Genetics **148:** 479-494.

Heitz, E. 1929. Heterochromatin, Chromozentren, Chromomeren. Ber. Dtsch. Bot. Ges. **47:** 274-284.

Hori, T., Takahashi, E., Tanigami, A., Tokino, T., and Nakamura, Y. 1992. A high resolution cytogenetic map of 168 cosmid DNA markers for human chromosome 11. Gemomics **13:** 129-133.

Hsu, T.C. 1979. Human and mammalian cytogenetics: an historical perspective. Springer-Verlag, New York.

Hulbert, S.H., Richter, T.E., Axtell, J.D., and Bennetzen, J.L. 1990. Genetic mapping and characterization of sorghum and related crops by means of maize DNA probes. Proc. Natl. Acad. Sci. USA **87:** 4251-4255.

Humphreys, M.W., Pasakinskiene, I., James, A.R., and Thomas, H. 1998. Physically mapping quantitative traits for stress-resistance in the forage grasses. J. Exp. Bot. **49:** 1611-1618.

Islam-Faridi, M.N., Childs, K.L., Klein, P.E., Hodnett, G., Menz, M.A., Klein, R.R., Rooney, W.L., Mullet, J.E., Stelly, D.M., and Price, H.J. 2002. A molecular cytogenetic map of sorghum chromosome 1: fluorescence *in situ* hybridization analysis with mapped bacterial artificial chromosomes. Genetics, **161:** 345-353.

Jackson, S.A., Wang, M.L., Goodman, H.M., and Jiang, J. 1998. Application of fiber-FISH in genome analysis of *Arabidopsis thaliana*. Genome **41:** 566-572.

Jackson, S.A., Cheng, Z.K., Wang, M.L., Goodman, H.M., and Jiang, J. 2000. Comparative fluorescence *in situ* hybridization mapping of a 431-kb *Arabidopsis thaliana* bacterial artificial chromosome contig reveals the role of chromosomal duplications in the expansion of the *Brassica rapa* genome. Genetics **156:** 833-838.

Jacobsen, E., de Jong, J.H., Kamstra, S.A., van den Berg, P.M.M.M., and Ramanna, M.S. 1995. Genomic *in situ* hybridization (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. Heredity **74:** 250-257.

Jewell, D.C., and Islam-Faridi, M.N. 1994. Details of a technique for somatic chromosome preparation and C-banding of maize. *In* The maize handbook. *Edited by* M. Freeling, and V. Walbot. Springer-Verlag, New York. pp. 484-493.

Ji, Y., Raska, D.A., McKnight, T.D., Islam-Faridi, M.N., Crane, C.F., Zwick, M.S., Hanson, R.E., Price, H.J., and Stelly, D.M. 1997. Use of meiotic FISH for identification of a new monosome in *Gossypium hirsutum* L. Genome **40:** 34-40.

Ji, Y., Raska, W.A., de Donato, M., Islam-Faridi, M.N., Price, H.J., and Stelly, D.M. 1999a. Identification and distinction segmental duplication-deficiencies by

fluorescence *in situ* hybridization (FISH)-adorned multivalent analysis. Genome **42:** 763-771.

Ji, Y., Stelly, D.M., de Donato, M., Goodman, M.M., and Williams, C.G. 1999b. A candidate recombination modifier gene for *Zea mays* L. Genetics **151:** 821-830.

Jiang, J., and Gill, B.S. 1994. Nonisotopic *in situ* hybridization and plant genome mapping: the first 10 years. Genome **37:** 717-725.

Jiang, J., Gill, B.S., Wang, G.L., Ronald, P.C., and Ward, D.C. 1995. Metaphase and interphase fluorescence *in situ* hybridization mapping of the rice genome with bacterial artificial chromosomes. Proc. Natl. Acad. Sci. USA **92:** 4487-4491.

Jiang, J., Hulbert, S.H., Gill, B.S., and Ward, D.C. 1996. Interphase fluorescence *in situ* hybridization mapping: a physical mapping strategy for plant species with large complex genomes. Mol. Gen. Genet. **252:** 497-502.

John, H.A., Birnstiel, M.L., and Jones, K.W. 1969. RNA-DNA hybrids at the cytological level. Nature **223:** 582-587.

Karpen, G.H., and Allshire, R.C. 1997. The case of epigenetic effects on centromere identity and function. Trends Genet. **13:** 489-496.

Kato, S., and Fukui, K. 1998. Condensation pattern (CP) analysis of plant chromosomes by an improved chromosome image analysing system, CHIAS III. Chromosome Research **6:** 473-479.

Kim, J.-S., Childs, K.L., Islam-Faridi, M.N., Menz, M.A., Klein, R.R., Klein, P.E., Price, H.J., Mullet, J.E., and Stelly, D.M. 2002. Integrated karyotyping of sorghum by *in situ* hybridization of landed BACs. Genome, **45:** 402-412.

Klein, P.E., Klein, R.R., Cartinhour, S.W., Ulanch, P.E., Dong, J., Obert, J.A., Morishige, D.T., Schlueter, S.D., Childs, K.L., Ale, M., and Mullet, J.E. 2000. A high-throughput AFLP-based method for constructing integrated genetic and physical maps: Progress toward a sorghum genome map. Genome Res. **10:** 789-807.

Klein, R.R., Klein, P.E., Chhabra, A.K., Dong, J., Pammi, S., Childs, K.L., Mullet, J.E., Rooney, W.L., and Schertz, K.F. 2001. Molecular mapping of the *rf1* gene for pollen fertility restoration in sorghum (*Sorghum bicolor* L.). Theor. Appl. Genet. **102:** 1206-1212.

Kong, L., Dong, J., and Hart, G.E. 2000. Characteristics, linkage-map positions, and allelic differentiation of *Sorghum bicolor* (L.) Moench DNA simple-sequence repeats (SSRs). Theor. Appl. Genet. **101:** 438-448.

Kulikova, O., Gualtieri, G., Geurts, R., Kim, D.-J., Cook, D., Huguet, T., de Jong, J.H., Fransz, P.F., and Bisseling, T. 2001. Integration of the FISH pachytene and genetic maps of *Medicago truncatula*. Plant J. **27:** 49-58.

Künzel, G., Korzun, L., and Meister, A. 2000. Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics **154:** 397-412.

Kurata, N., Umehara, Y., Tanoue, H., and Sasaki, T. 1997. Physical mapping of rice genome with YAC clones. Plant Mol. Biol. **35:** 101-113.

Lapitan, N.L.V., Ganal, M.W., and Tanksley, S.D. 1989. Somatic chromosome karyotype of tomato based on *in situ* hybridization of the TGRI satellite repeat. Genome **32:** 992-998.

Lichter, P., Tang, C.J., Call, K., Hermanson, G., Evans, G.A., Housman, D., and Ward, D.C. 1990. High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science **247:** 64-69.

Masterson, J. 1994. Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science (Washington, D.C.) **264:** 421-423.

Matern, B., and Simak, M. 1968. Statistical problems in karyotype analysis. Hereditas (Lund, Swed.) **59:** 280-288.

McClintock, B. 1930. A cytological demonstration of the location of an interchange between two non-homologous chromosomes of *Zea ma*ys. Proc. Nat. Acad. Sci. USA **16:** 791-796.

McCombie, W.R., de la Bastide, M., Habermann, K., Parnell, L., Dedhia, N., Gnoj, L., Schutz, K., Huang, E., Spiegel, L., Yordan, C., Sehkon, M., Murray, J., Sheet, P., Cordes, M., Threideh, J., Stoneking, T., Kalicki, J., Graves, T., Harmon, G., Edwards, J., Latreille, P., Courtney, L., Cloud, J., Abbott, A., Scott, K., Johnson, D., Minx, P., Bentley, D., Fulton, B., Miller, N., Greco, T., Kemp, K., Kramer, J., Fulton, L., Mardis, E., Dante, M., Pepin, K., Hillier, L., Nelson, J., Spieth, J., Simorowski, J., May, B., Ma, P., Preston, R., Vil, D., See, L.H., Shekher, M., Matero, A., Shah, R., Swaby, I., O'Shaughnessy, A., Rodriguez, M., Hoffman, J., Till, S., Granat, S., Shohdy, N., Hasegawa, A., Hameed, A., Lodhi, M., Johnson, A., Chen, E., Marra, M., Wilson, R.K., and Martienssen, R. 2000. The complete sequence of a heterochromatic island from a higher eukaryote. Cell **100:** 377-386.

Menz, M.A., Klein, R.R., Mullet, J.E., Obert, J.A., Unruh, N.C., and Klein, P.E. 2002. A

high-density genetic map of *Sorghum bicolor* (L.) Moench based on 2926 AFLP, RFLP and SSR markers. Plant Mol Biol. **48:** 483-499.

Moir, D.T., Dorman, T.E., Day, J.C., Ma, N.S., Wang, M., and Mao, J. 1994. Toward a physical map of human chromosome 10: isolation of 183 YACs representing 80 loci and regional assignment of 94 YACs by fluorescence *in situ* hybridization. Genomics **22:** 1-12.

Moore, G., Devos, K.M., Wang, Z., and Gale, M.D. 1995. Grasses, line up and form a circle. Current Biology **5:** 737-739.

Morishige, D.T., Childs, K.L., Moore, L.D., and Mullet, J.E. 2002. Targeted analysis of orthologous *phytochrome A* regions of the sorghum, maize, and rice genomes using comparative gene-island sequencing. Plant Physiology **130:** 1614-1625.

Muleris, M., Apiou, F., Olschwang, S., Thomas, G., and Dutrillaux, B. 1994. Mapping of 18 probes on human chromosome 18 using single- and double-color FISH. Cytogenetics & Cell Genetics **65:** 82-85.

Peng, Y., Schertz, K.F., Cartinhour, S., Hart, G.E. 1999. Comparative genome mapping of *Sorghum bicolor* (L.) Moench using an RFLP map constructed in a population of recombinant inbred lines. Plant Breed. **118:** 225-235.

Pereira, M.G., Lee, M., Bramel-Cox, P., Woodman, W., Doebley, J., Whitkus, R. 1994. Construction of an RFLP map in sorghum and comparative mapping in maize. Genome **37:** 236-243.

Peterson, D.G., Price, H.J., Johnson, J.S., and Stack, S.M. 1996. DNA content of heterochromatin and euchromatin in tomato (*Lycopersicon esculentum*) pachytene chromosomes. Genome **39:** 77-82.

Peterson, D.G., Lapitan, N.L.V., and Stack, S.M. 1999. Localization of single- and low-copy sequences on tomato synaptonemal complex spreads using fluorescence *in situ* hybridization (FISH). Genetics **152:** 427-439.

Powell, W., Thomas, W.T.B., Baird, E., Lawrence, P., Booth, A., Harrower, B., McNicol, J.W., and Waugh, R. 1997. Analysis of quantitative traits in barley by the use of amplified fragments length polymorphisims. Heredity **79**: 48-59.

Putterill, J., Robson, F., Lee, K., and Coupland, G. 1993. Chromosome walking with YAC clones in *Arabidopsis*: isolation of 1700 kb of contiguous DNA on chromosome 5, including a 300 kb region containing the flowering-time gene *CO*. Mol. Gen. Genet. **239:** 145-157.

Qi, X., Stam, P., and Lindhout, P. 1998. Use of locus-specific AFLP markers to construct a high-density molecular map in barley. Theor. Appl. Genet. **96:** 376-384.

Quinby, J.R. 1967. The maturity genes of sorghum. *In* Advances in agronomy. Vol. 19. *Edited by* A.G. Norman. Academic Press, New York. pp. 267-305.

Quinby, J.R. 1974. Sorghum Improvement and the Genetics of Growth. Texas A&M University Press, College Station.

Reinisch, A.J., Dong, J.M., Brubaker, C.L., Stelly, D.M., Wendel, J.F., and Paterson, A.H. 1994. A detailed RFLP map of cotton, *Gossypium hirsutum* × *Gossypium barbadense*: chromosome organization and evolution in a disomic polyploidy

genome. Genetics **138:** 829-847.

Reyes-Valdes, M.H., Ji, Y., Crane, C.F., Talyor, J.F., Islam-Faridi, M.N., Price, H.J., and Stelly, D.M. 1996. ISH-facilitated analysis of meiotic bivalent pairing. Genome **39:** 784-792.

Riera-Lizarazu, O., Vales, M.I., Ananiev, E.V., Rines, H.W., and Phillips, R.L. 2000. Production and characterization of maize-chromosome 9 radiation hybrids derived from an oat-maize chromosome addition line. Genetics **156:** 327-339.

Sadder, M.T., Ponelies, N., Born, U., and Weber, G. 2000. Physical localization of single-copy sequences on pachytene chromosomes in maize (*Zea mays* L.) by chromosome *in situ* suppression hybridization. Genome **43:** 1081-1083.

Salih, A.A., Ali, I.A., Lux, A., Luxova, M., Cohen, Y., Sugimoto, Y., and Inanaga, S. 1999. Rooting, water uptake, and xylem structure adaption to drought of two sorghum cultivars. Crop Sci. **39:** 168-173.

Sang, Y.J., and Liang, G.H. 2000. Comparative physical mapping of the 18S-5.8S-26S rDNA in three sorghum species. Genome **43:** 918-922.

SanMiguel, P., Tikhonov, A., Jin, Y.-K., Motchoulskaia, N., Zakharov, D., Melake-Berhan, A., Springer, P.S., Edwards, K.J., Lee, M., Avramova, Z., and Bennetzen, J.L. 1996. Nested retrotransposons in the intergenic regions of the maize genome. Science **274:** 765-768.

SanMiguel, P., and Bennetzen, J.L. 1998. Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. Ann. Bot. **82:** 37-44.

Schertz, K.F., and Dalton, L.G. 1980. Sorghum. *In* Hybridization of crop plants. *Edited by* W. Fehr, and H.H. Hadly. Am. Soc. Agron., Crop Sci. Soc. Of America, Madison, Wisconsin. pp. 577-588.

Schertz, K.F., Sotomayor-Rios, A., and Torres-Cardona, S. 1989. Cytoplasmic-nuclear male sterility: opportunities in breeding and genetics. Proc. Grain Sorghum Res. And Utility Conf. **16:** 175-186.

Schmidt, R., West, J., Love, K., Lenehan, Z., Lister, C., Thompson, H., Bouchez, D., and Dean, C. 1995. Physical map and organization of *Arabidopsis thaliana* chromosome 4. Science **270:** 480-483.

Schmidt, T., and Heslop-Harrison, J.S. 1998. Genomes, genes and junk: the large-scale organization of plant chromosomes. Trends Plant Sci. **3:** 195-199.

Shen, D.L., Wang, Z.F., and Wu, M. 1987. Gene mapping on maize pachytene chromosomes by *in situ* hybridization. Chromosoma **95:** 311-314.

Sherman, J.D., and Stack, S.M. 1995. Two-dimensional spreads of synaptonemal complexes from solanaceous plants. VI. High-resolution recombination nodule map for tomato (*Lycopersicon esculentum*). Genetics **141:** 683-708.

Song, J., Dong, F., and Jiang, J. 2000. Construction of a bacterial artificial chromosome (BAC) library for potato molecular cytogenetics research. Genome **43:** 199-204.

Stebbins, G.L. 1950. Variation and evolution in plants. Columbia University Press: New York.

Stelly, D.M., Crane, C.F., Hanson, R.E., McKnight, T.D., and Price, H.J. 1996. Meiotic molecular cytogenetics of cotton. *In* Methods of genome analysis in plants: their

merits and pitfalls. *Edited by* P.P. Jauhar. CRC Press. pp. 99-111.

Takahashi, E., Yamakawa, K., Nakamura, Y., and Hori, T. 1992. A high resolution cytogenetic map of human chromosome 3: localization of 291 new cosmid markers by direct R-banding fluorescence *in situ* hybridization. Genomics **13:** 1047-1055.

Tanksley, S.D., Ganal, M.W., Prince, J.P., de Vicente, M.C., and Bonierbale, M.W., Broun, P., Fulton, T.M., Giovannoni, J.J., Grandillo, S., Martin, G.B., Messeguer, R., Miller, J.C., Miller, L., Paterson, A.H., Pineda, O., Roder, M.S., Wing, R.A., Wu, W., and Young, N.D. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics **132:** 1141-1160.

Tao, Q., and Zhang, H.B. 1998. Cloning and stable maintenance of DNA fragments over 300 kb in *Escherichia coli* with conventional plasmid-based vectors. Nucl. Acids Res. **26:** 4901-4909.

Tao, Q., Chang, Y., Wang, J., Chen, H., Islam-Faridi, M.N., Scheuring, C., Wang, B., Stelly, D.M., and Zhang, H.B. 2001. BAC-based physical map of the rice genome constructed by restriction fingerprint analysis. Genetics **158:** 1711-1724.

Tarchini, R., Biddle, P., Wineland, R., Tingey, S., and Rafalski, A. 2000. The complete sequence of 340 kb of DNA around the rice *Adh1-Adh2* region reveals interrupted colinearity with maize chromosome 4. Plant Cell **12:** 381-391.

Tikhonov, A.P., SanMiguel P.J., Nakajima, Y., Gorenstein, N.M., Bennetzen, J.L., and Avramova, Z. 1999. Colinearity and its exceptions in orthologous *adh* regions of maize and sorghum. Proc. Natl. Acad. Sci. USA **96:** 7409-7414.

Trask, B.J. 1991. Fluorescence *in situ* hybridization: applications in cytogenetics and

gene mapping. Trends Genet. **7:** 149-154.

Walter, M.A., Spillet, D.J., Thomas, P., Weissienbach, J., Goodfellow, P.N. 1994. A method for constructing radiation hybrid maps of whole genomes. Nature Genetics 7: 22-28.

Weber, D., and Helentjaris, T. 1989. Mapping RFLP loci in maize using B-A translocations. Genetics **121:** 583-590.

Werner, J.E., Endo, T.R., and Gill, B.S. 1992. Toward a cytogenetically based physical map of the wheat genome. Proc. Natl. Acad. Sci. USA **89:** 11307-11311.

Woo, S.S., Jiang, J., Gill, B.S., Paterson, A.H., and Wing, R.A. 1994. Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*. Nucl. Acids Res. **22:** 4922-4931.

Wu, T.P. 1992. B-chromosomes in *Sorghum stipoideum*. Heredity **68:** 457-463.

Xu, G.W., Magill, C.W., Schertz, K.F., and Hart, G.E. 1994. An RFLP linkage map of *Sorghum bicolor* (L.) Moench. Theor. Appl. Genet. **89:** 139-145.

Xu, J., and Earle, E.D. 1996. High resolution physical mapping of 45S (5.8S, 18S and 25S) rDNA gene loci in the tomato genome using a combination of karyotyping and FISH of pachytene chromosomes. Chromosoma **104:** 545-550.

Yu, H., Liang, G.H., and Kofoid, K.D. 1991. Analysis of C-banding chromosome patterns of sorghum. Crop Sci. **31:** 1524-1527.

Zhong, S.B., de Jong, J.H., and Zabel, P. 1996. Preparation of tomato meiotic pachytene and mitotic metaphase chromosomes suitable for fluorescence *in situ* hybridization (FISH). Chromosome Res. **4:** 24-28.

Zhong, X.B., Bodeau, J., Fransz, P.F., Williamson, V.M., and van Kammen, A., de Jong, J.H., and Zabel, P. 1999. FISH to meiotic pachytene chromosomes of tomato locates the root knot nematode resistance gene *Mi-1* and the acid phosphatase gene *Aps-1* near the junction of euchromatin and pericentromeric heterochromatin of chromosome arms 6S and 6L, respectively. Theor. Appl. Genet. **98:** 365-370.

Zwick, M.S., Islam-Faridi, M.N., Czeschin, Jr. D.G., Wing, R.A., Hart, G.E., Stelly, D.M., and Price, H.J. 1998. Physical mapping of the *liguleless* linkage group in *Sorghum bicolor* using rice RFLP-selected sorghum BACs. Genetics **148**: 1983-1992.

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