The Plant Journal (2003) **35**, 647–659 doi: 10.1046/j.1365-313X.2003.01829.x

TECHNICAL ADVANCE

A new single-locus cytogenetic mapping system for maize (*Zea mays* L.): overcoming FISH detection limits with marker-selected sorghum (*S. propinquum* L.) BAC clones

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Summary

The development of a cytogenetic map for maize (*Zea mays* L.) is shown to be feasible by means of a combination of resources from sorghum and oat that overcome limitations of single-copy gene detection. A maize chromosome-addition line of oat, OMAd9.2, provided clear images of optically isolated pachytene chromosomes through a chromosome spread and painting technique. A direct labeled oligonucleotide fluorescence *in situ* hybridization (FISH) probe MCCY specifically stained the centromere. The arm ratio (long/short) for maize chromosome 9 in the addition line was 1.7, comparable to the range of 1.6–2.1 previously reported for maize chromosome 9. A sorghum (*Sorghum propinquum* L.) BAC library was screened by hybridization with each of three maize core-bin-marker (CBM) probes: umc109 (CBM9.01), umc192/bz1 (CBM9.02), and csu54b (CBM9.08). A single BAC clone for each marker was chosen; designated sCBM9.1, sCBM9.2, or sCBM9.8; and used as a FISH probe on pachytene spreads from OMAd9.2. In each case, discrete FISH signals were observed, and their cytogenetic positions were determined to be 9S.79 (at position 79% of the length of chromosome 9 short arm) for sCBM9.1, 9S.65 for sCBM9.2, and approximately 9L.95 for sCBM9.8. These map positions were co-linear with linkage-map positions for these and other loci common to the linkage and cytogenetic maps. This work represents a major breakthrough for cytogenetic mapping of the maize genome, and also provides a general strategy that can be applied to cytogenetic mapping of other plant species with relatively large and complex genomes.

Keywords: maize, cytogenetic, pachytene, FISH, sorghum, genomics.

Introduction

The structure and function of the maize (*Zea mays* L.) genome has been examined cytologically for more than 70 years (Carlson, 1988; Rhoades and McClintock, 1935). Microscopic analysis of maize meiotic chromosomes has yielded numerous and historic contributions to our understanding of basic principles of genetics such as the biology of transposons, telomeres, and recombination (Creighton and McClintock, 1931; McClintock, 1931, 1941, 1978). Despite the rich history of maize cytogenetics, the physical location of the maize genes remains largely unknown except for some indirect determinations from genetically marked translocation breakpoints (Harper and Cande, 2000). The genetic linkage maps for maize are, by contrast, very well developed, consisting of thousands of loci including classical mutation loci, restriction fragment length polymorphism (RFLP) markers, simple sequence-repeat (SSR) markers, quantitative trait locus (QTL) markers, and expressed sequence tag (EST) markers (Davis *et al.*, 1999; Lee *et al.*, 2002; Sharopova *et al.*, 2002). In addition, more than 150 000 maize ESTs have been produced, and a recent assembly of 73 000 of them allowed identification of more than 20 000 'tentative unique genes' (TUGs; Fernandes *et al.*, 2002).

With the advent of functional genomics, genome-sequencing projects, and physical map development for maize, a cross-linked cytogenetic map for maize could provide useful and interesting knowledge for genomics research.

Well-developed cytogenetic maps for *Drosophila* and human are known for their usefulness in many aspects of basic and applied research (Lozovskaya *et al.*, 1993; Trask, 2002). Maize research is also characterized by large components of basic and applied research (Sprague and Dudley, 1988). Recent research has focused on the use of maize for comparative genomics (Devos and Gale, 1997, 2000; Freeling, 2001; Gale and Devos, 1998; Ware *et al.*, 2002; Wilson *et al.*, 1999), as a model grass for studies of genome evolution (Gaut, 2001; Gaut *et al.*, 2000), and as a model genetic organism for a new genome sequencing initiative (Bennetzen *et al.*, 2001).

The maize karyotype and cytogenetic maps are based on the pachytene chromosome, the long synapsed chromosome fiber unique to meiotic prophase. The units of the cytogenetic map in maize are based on the fractional distance along the long or short arm, so the cytogenetic map is constant and easy to integrate with data going back to the earliest studies (Carlson, 1988). Sadder and Weber (2001) have also developed a fluorescence *in situ* hybridization (FISH) karyotype of maize based on mitotic chromosomes, which have inherently less axial resolution and fewer links to the classical cytological data than do meiotic chromosomes. The pachytene cytogenetic map has nonetheless remained underdeveloped for decades (Rhoades, 1950). The more recent cytogenetic maps are annotated with loci such as knobs, centromeres, FISH-detectable tandem repeat clusters, and translocation break points but virtually devoid of single-gene loci except for three genes on chromosome 9 (Coe, 1994; Sadder and Weber, 2002; Sadder *et al.*, 2000; Shen *et al.*, 1987).

In recent years, fluorescent imaging technologies have been applied to the examination of meiotic pachytene-stage chromosomes in many different plant species (Gill and Friebe, 1998; de Jong *et al.*, 1999; Lysak *et al.*, 2001). High-resolution FISH in maize has been used to visualize and analyse specific DNA segments on pachytene-stage chromosomes from pollen mother cells (Bass *et al.*, 1997, 2000b, 2003; Carlton and Cande, 2002), but single-gene FISH has remained difficult in maize for two reasons: the inherent detection limit of a few kilobase pair for routine FISH; and the abundance of intragenic retrotransposon sequences that can give erroneous cross-hybridization to multiple loci. Even large segments of maize genomic DNA, therefore typically contain only a few kilobase pairs of single-copy gene sequences and do not overcome the detection limit, except for carefully chosen large clones in combination with suppression hybridization (Sadder and Weber, 2002; Sadder *et al.*, 2000). However, a solution to both the gene-density and the intragenic repetitive-DNA problems may come from use of genomic clones selected from sorghum ($2n = 2x = 20$), a diploid relative of maize with a relatively small genome of 0.7×10^9 to 0.8×10^9 Mbp (Arumuganathan and Earle, 1991). The genic portions of the sorghum and maize genomes are co-linear and are highly conserved at the DNA sequence level, but sorghum has a three- to fourfold higher gene density and lacks the extensive intragenic retrotransposons characteristic of maize (Gaut, 2001; Gaut *et al.*, 2000). More specifically, Zwick *et al.* (1998) demonstrated that sorghum BACs can be used as FISH probes on maize chromosomes. In addition, Woo *et al.* (1994) and Islam-Faridi *et al.* (2002) have clearly demonstrated the power of sorghum BACs as reagents for molecular cytology and mapping in sorghum. The availability of gridded-filter BAC libraries from sorghum has further permitted efficient use of sorghum genomic clones for analysis of homologous regions of the maize genome (Draye *et al.*, 2001; Lin *et al.*, 1999).

Here, we describe the use of the maize 9 chromosome-addition line of oat, OMAd9.2, in conjunction with maize-marker-selected sorghum BAC probes to develop a novel single-locus cytogenetic mapping strategy for the maize genome. Using three loci to test this approach, we show that sorghum BACs can be used as FISH probes to localize sequences on the pachytene chromosomes of maize. This strategy overcomes a technical barrier to the development of an anchored cytogenetic map for maize and could be adapted for use with other plant species for which large complex genomes limit prospects for FISH-based cytogenetic map development.

Results

In an effort to advance cytogenetic mapping of the maize genome, we used an alien chromosome-addition line, OMAd9.2, taking advantage of several unique features of this material (Bass *et al.*, 2000b; Kynast *et al.*, 2001). First, the entire maize chromosome 9 can be fluorescently painted by genomic *in situ* hybridization (GISH), providing clear detection of the entire chromosome fiber from end to end (Bass *et al.*, 2000b). Secondly, this single chromosome-addition line eliminates all chromosome identification problems. Finally, the addition line is more amenable to year-round harvest of pollen mother cells than are maize lines such as KYS, an inbred line, commonly used for cytogenetics.

The maize chromosome 9 arm ratio is preserved in the oat genome

In order to develop a cytogenetic mapping system that takes advantage of the simplifying features of single-chromosome addition lines, we first wanted to verify that maize chromosome 9 in the oat genome was similar to maize chromosome 9 in its natural maize genome background. So, we employed a 3D acrylamide FISH technique known to preserve the nuclear architecture and chromatin morphology in fixed pollen mother cells (Bass *et al.*, 1997, 2000b, 2003). Because the maize 9 pachytene chromosome

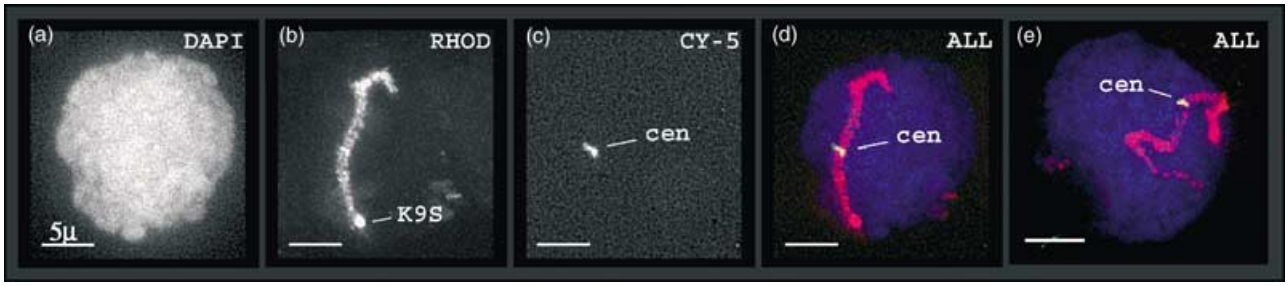

Figure 1. 3D acrylamide FISH of maize chromosome 9 paint and the centromere. Pachytene-stage PMCs from OMAd9.2 were fixed in formaldehyde, embedded in acrylamide, and subjected to FISH and 3D imaging (Bass *et al.*, 1997). Image datasets were cropped and displayed with the maximum-intensity through-focus projection scheme. Individual wavelength names (DAPI, RHOD, and CY-5) refer to the filter sets used and are shown separately as gray-scale images or in combination as pseudocolor overlay images (ALL).
(a) The DAPI image shows total chromatin in the nucleus.
(b) The rhodamine channel image (RHOD) shows chromosome paint signals from the GISH probe of direct labeled maize total DNA and illustrates the bright optical clarity characteristic of this system (Bass *et al.*, 2000b). The small knob at the end of the short arm (K9S) is often evident even with knobless DNA as a GISH probe.
(c) The Cy5 image (CY-5) shows the bright discrete centromere FISH signal (cen) from the oligonucleotide probe MCCY.
(d) Three-color overlay images show the combination of the DAPI (blue), rhodamine (red), and Cy5 (green) data from the previous panels (a–c). The position of the maize chromosome 9 within the nucleus is seen together with the location of centromere (cen, appears yellow from red-plus-green overlap).
(e) Example of another nucleus, displayed as described for panel (d). Scale bars are 5 μm.

is already well characterized, we compared our arm-ratio data to those from other studies to determine whether the maize chromosome 9 in OMAd9.2 showed any evidence of being misfolded or irregularly organized. We developed a centromere-detecting oligonucleotide probe to mark the centromere and painted the maize chromosome 9 by maize GISH in fixed OMAd9.2 pachytene-stage cells.

Pachytene-stage pollen mother cells (PMCs) were subjected to 3D FISH and multiple-wavelength image collection. Two representative examples are shown in Figure 1 as through-focus projections of the entire nucleus. The 4′,6-diamidino-2-phenylindole (DAPI) image (Figure 1a, DAPI) shows total chromatin, which appears as a solid mass in this projection but is made up of thick pachytene fibers that are evident in single optical sections (not shown). The maize chromosome 9 fiber is visualized in the rhodamine channel image (RHOD, Figure 1b) that results from FISH with rhodamine-labeled total maize DNA from the inbred line Knobless Tama Flint (KTF). The small knob at the end of the short arm and the doubled nature of the synapsed homologs are often evident in such images. The specific marking of the centromere (Figure 1c, CY-5, and 'cen') results from hybridization with the fluorescent-labeled oligonucleotide probe MCCY, designed to span a 25-bp region between two CentC repeats (Ananiev *et al.*, 1998). The maize GISH and the MCCY probes appear to preferentially hybridize to maize but not to any appreciable degree to the oat genome, indicating that these probes are essentially species-specific under these conditions.

Using computer-assisted chromosome modeling and tracking of images such as these, we determined a value of 1.7 for the average arm ratio (long/short) for the maize chromosome 9 in OMAd9.2. This value, as shown in Table 1, is similar to those determined by others for the maize chromosome 9 for the somatic or meiotic chromosomes that had been spread or squashed. The arm ratio in OMAd9.2 was independent of total length (not shown) and indicates that this material should be suitable for obtaining cytogenetic mapping data.

Maize core-bin-anchor probe-selected sorghum BACs

In order to overcome the limitations associated with using genomic clones from maize as FISH probes, we turned to a

Table 1 Maize chromosome 9 arm ratios

Ratio (L/S)[a]	Maize line	Cell type[b]	Reference
2.0	KYS	mei	Neuffer *et al.* (1997)
1.93 ± 0.34	KYS	mei	Chen *et al.* (2000)
2	KYS	som	Sadder and Weber (2001)
1.64	Seneca 60	som	Chen (1969)
1.9	KYS	mei	R.K. Dawe[c]
1.67 ± 0.07	OMAd9.2 (Seneca 60)	mei	This study: 3D acrylamide method
1.71 ± 0.03	OMAd9.2 (Seneca 60)	mei	This study: pachytene spreads

[a](L/S): long- to short-arm ratio.
[b]mei: meiotic prophase stage; som: somatic mitosis stage.
[c]Personal communication (in Dawe *et al.* (1992) *Maize Genetics Cooperative Newsletter* 66, 23–25).

Table 2 *Sorghum propinquum* BAC clones selected by hybridization with maize RFLP probes

Core-bin-marker[a]	Maize RFLP probes	Sorghum BACs detected[b]	Sorghum BAC clones obtained	BAC clones used as FISH probes[c]
CBM9.1	p-umc109	a0055A15 a0055B21 a0064C07 a0004M18 a0068L03 a0085L22 a0040A04	a0064C07 a0004M18	a0004M18 (sCBM9.1)
CBM9.2 (bz1)	p-umc192	a0028C12 a0020G06	a0028C12 a0020G06	a0020G06 (sCBM9.2)
CBM9.8	p-csu054	a0021O17 a0061D07 a0074A03 a0080B23 a0075H02	a0061D07 a0074A03	a0074A03 (sCBM9.8)

[a]Core-bin-markers are from UMC 98 linkage map (Davis *et al.*, 1999).
[b]Clones were identified from two filters containing 36 000 gridded BACs for CBM9.1 and CBM9.8 or for one filter containing 18 000 gridded BACs for CBM9.2.
[c]BAC names used for this study are in parentheses.

closely related species with a high level of genome co-linearity, sorghum (Gaut, 2001; Song *et al.*, 2002; Tikhonov *et al.*, 1999). On the basis of gene density and general lack of cross-hybridizing intergenic sequences, we reasoned that a single sorghum BAC clone would have a high likelihood of producing a discrete, detectable, and locus-specific FISH signal on the appropriate maize chromosome. We obtained plasmids for maize RFLP probes and used the inserts to screen a library of 36 000 sorghum BAC clones (*S. propinquum* library, from A. H. Paterson, University of Georgia, Athens, GA, USA) arrayed on a nylon filter. For this study, the core-bin-marker (CBM) loci were chosen because they are widely used genetic markers that are publicly available as plasmid clones. Hybridization (approximately $T_m - 12^\circ C$) of the radiolabeled maize marker probes resulted in the identification of several different sorghum BACs per probe (Table 2), consistent with the sixfold coverage of the library filter set. Marker-selected groups of clones belonged to fingerprint contigs (FPCs) that were independently identified with overgo probes (A. H. Paterson and J. E. Bowers, personal communication). We obtained two overlapping BACs for each CBM and chose one for each CBM to use as a FISH probe (Table 2) for subsequent experiments. The presence of the RFLP cross-hybridizing sequences was verified by Southern blot analysis as shown in Figure 2. Each maize RFLP insert

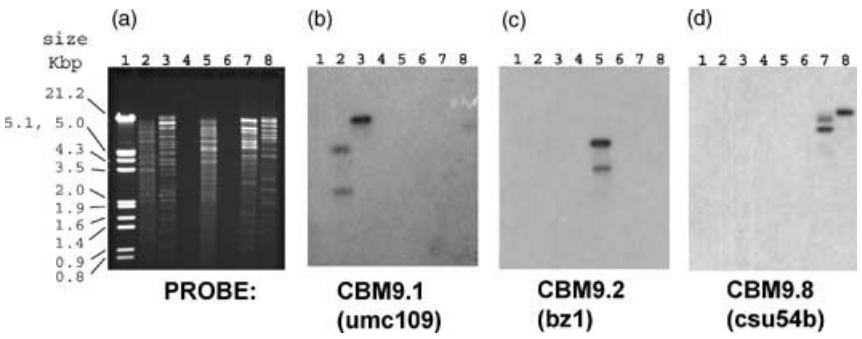

Figure 2. Southern blot verification of markers. The Southern blot technique was used to verify that the sorghum BAC clones obtained were the correct clones, as indicated by hybridization with radiolabeled probes for the maize RFLP markers CBM9.1, CBM9.2, and CBM9.8 (see Table 2).
(a) An agarose gel of restriction enzyme-digested sCBM BAC clones stained with ethidium bromide after electrophoresis and before transfer. Lanes: 1, *Hin*dIII/*Eco*RI lambda DNA marker; 2, *Eco*RI sCBM9.1; 3, *Hin*dIII sCBM9.1; 4, blank; 5, *Eco*RI sCBM9.2; 6, blank; 7, *Eco*RI sCBM9.8; 8, *Hin*dIII sCBM9.8. The DNA on the blot was subjected to sequential hybridization, exposure, and stripping with probes from isolated inserts corresponding to CBM9.1 (b), CBM9.2 (c), or CBM9.8 (d). Sizes (kbp) of the marker bands are indicated at the left.

hybridized to its corresponding BAC clone producing one or two bands per restriction digest (Figure 2b–d). Here, we have renamed the chosen sorghum BAC clones sCBM9.1, sCBM9.2, and sCBM9.8 for core markers 9.01, 9.02, and 9.08, respectively.

Previously, we have attempted to detect a single locus with the acrylamide FISH method (Bass *et al.*, 1997) along with post-hybridization signal-amplification schemes, but the results were irregular and unsatisfactory. By combining a chromosome spreading technique with indirect immunofluorescent detection of hybridized, labeled sorghum BAC DNA, however, we obtained discrete signals that were clearly evident in the initial images, even before deblurring with 3D deconvolution. This new approach did not adversely affect the preservation of the arm ratios (Table 1). We, therefore, proceeded to map the three loci that were chosen for several reasons: to represent each arm, to represent the last locus in the linkage group, and to represent at least one locus for which extensive cytological, genetic, and molecular data were known, the *Bronze1*/CBM9.2 locus (Dooner and Martinez-Ferez, 1997; Fu and Dooner, 2000, 2002; Fu *et al.*, 2001, 2002; Neuffer *et al.*, 1997).

sCBM9.2 FISH signals map to cytogenetic position 9S.65

The maize core-bin-anchor probe 9.02 is the boundary marker separating the linkage bins 9.01 and 9.02. By convention, it is part of the linkage group of 9.02, and is the first locus in that bin from the telomere end of the short arm. Here, we refer to this marker as CBM9.2 to distinguish it clearly from bin 9.02 to which it belongs. Likewise, CBM9.3 refers to core-bin-anchor probe 9.03 (p-umc25); and it would be the first locus following those in bin 9.02, and so on. A clone for CBM9.2 is available as 'p-umc192', a plasmid carrying a 1.75-kbp insert from a cDNA clone of *Bronze1* (*Bz1*). Of importance to the present study, the cytogenetic position of *Bz1* has been approximated from analysis of chromosome translocation stocks (discussed below), so it provides a means to check the validity of any FISH-based map data for sCBM9.2 in OMAd9.2.

In all of the FISH experiments involving the CBMs, 3D four-wavelength images were obtained. The co-hybridization mix contained the FISH probe MCCY for detection of the centromere in the Cy-5 channel, the Alexa488-labeled maize total DNA for detection of the entire maize chromosome 9 in the flourescein isothiocyanate (FITC) channel, and digoxigenin-labeled BAC DNA. The digoxigenin was visualized by means of a fluorescent antibody enhancement set in which the FITC-conjugated antibodies were replaced at the same concentration with rhodamine-conjugated antibodies. After the indirect labeling, the slides were counter-stained with DAPI, mounted, and were photographed by 3D deconvolution microscopy as previously described (Bass *et al.*, 2000a). Each dataset consists of optical reconstructions in which the DAPI, FITC, rhodamine, and Cy-5 separately record the total DNA, maize chromosome 9 fiber, BAC signals, and centromere, respectively.

The entire sCBM9.2 BAC clone (a0020G06, Table 2) was labeled with digoxigenin-dUTP and was used as a FISH probe. Examples of the resulting image data are shown in Figure 3. Comparison of the DAPI and FITC images (Figure 3a,b) reveals the specificity of the maize paint and uniform end-to-end labeling that allows the path of the whole chromosome to be clearly visualized. In addition, the knob that is commonly found at the end of the 9S is evident in the DAPI image but not in the paint, which was prepared from a knobless line of maize. The MCCY probe consistently hybridizes to the primary constriction of maize chromosome 9, marking the centromere (Figure 3c and 'cen'). A pair of bright FISH signals was observed in the rhodamine channel (Figure 3d, sCBM9.2), present on the short arm of maize chromosome 9, and significantly above background staining levels. A three-color overlay (Figure 3e) shows the position of the FISH signals on the fiber (red for paint, blue for centromere, and green for sCBM9.2). For cytogenetic mapping purposes, we employed computer-assisted chromosome straightening. Three different straightened chromosomes are shown in Figure 3(f); the top one corresponds to the same chromosome shown in Figure 3(e). The green spots (Figure 3e,f; sCBM9.2) represent FISH signals that we attribute to hybridization of sCBM9.2 to homologous sequences on the maize chromosome 9. The FISH signals were located at an averaged position of 65% of the distance down the short arm, namely cytogenetic position 9S.65.

The mapping data for sCBM9.2 represent the average value from 56 chromosomes collected from four different slides representing two separate experiments. Examination of the images revealed that spots could be found all over the slide, and these spots had morphologies that overlapped with what we considered to be the true FISH signals. Generally, the FISH signals can be identified by a combination of criteria including (i) reproducible localization, (ii) their relative brightness, and (iii) the frequent doublets or multiple spots that probably result from hybridization to the pachytene stage (synapsed) homologs. To check a worst-case scenario, we determined the positions of all the spots in the rhodamine channel that could be interpreted as near the fiber, pooled them by bins of 10% of the length of the short arm, and plotted their numbers (Figure 3g). We obtained a total of 192 signals in this way, without excluding any for not being doublets or for having good spatial overlap with the fiber. For comparison, we carried out randomization tests with nine more 192-point samples and plotted those results in Figure 3(g). The unique spike from the actual data (sCBM9.2) provided an additional measure of confidence in our spot-calling decisions. Subsequent experiments in which excess unlabeled sorghum

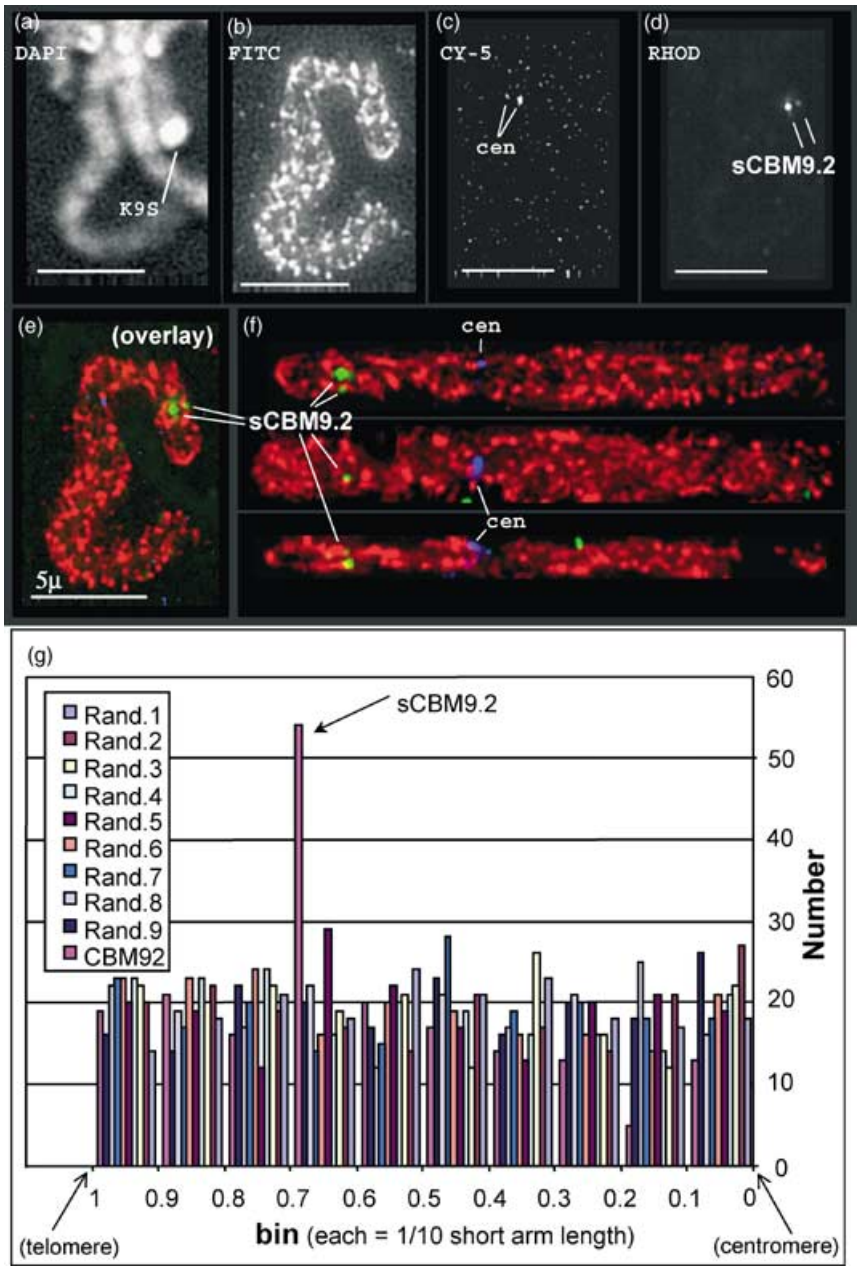

Figure 3. Localization of FISH signals from sCBM9.2. Pachytene-stage PMCs were fixed, spread, and treated for FISH. 3D images are displayed as described for Figure 1. Single-wavelength images for one example are shown in panels (a–d), and the overlay images represent maize chromosome 9 as seen before (e) or after (f) computer-assisted chromosome path straightening (f).
(a) The DAPI image shows the partial view of the edge of a single nucleus, revealing the oat and maize thick pachytene fibers. The terminal knob on maize chromosome 9, which is more clearly evident in spread preparations, is indicated (K9S).
(b) The FITC image shows maize chromosome 9 paint from A488-dUTP-labeled maize DNA.
(c) The Cy5 image shows centromere FISH signals (cen).
(d) The rhodamine image shows indirect immunofluorescence signals (sCBM9.2) from digoxigenin-labeled sCBM9.2 BAC DNA.
(e) RGB pseudocolor overlay of the FITC (red), Cy-5 (blue), and rhodamine (green) images reveal the position of the centromere and sCBM9.2 signals on the maize 9 fiber.
(f) Color overlay of projections of straightened chromosomes from three different nuclei (top one is same as (e)). The chromosomes are arranged with the short arm at the left. The location of FISH signals for the centromere (cen) and sCBM9.2 are indicated.
(g) All spots that could be interpreted as FISH signals on 9S were mapped and plotted along with nine randomized samples (Rand.1–9) for comparison. A total of 192 spots were mapped to a cytogenetic coordinate (the fractional distance along the arm, where the centromere is 0 and the telomere is 1.0) and pooled into bins corresponding to 1/10 of the arm length. The number of signals in each bin is plotted, revealing the strong signal above background for sCBM9.2. All scale bars are 5 μm.

DNA was included as a blocking reagent further reduced the frequency of scattered background spots (not shown).

sCBM9.8 FISH signals map to cytogenetic position 9L.95

Next, we carried out FISH with sCBM9.8, representing the most terminal locus on the UMC-1998 maize linkage map (Davis *et al.*, 1999). Chromosome images were collected, deconvolved, modeled, straightened, and analysed as above; examples are shown in Figure 4. The FISH signals analysed from 56 chromosomes were found to localize to one or two locations near the tip of the long arm of maize 9, always between 9L.90 and 9L.99. This cytogenetic position is consistent with sCBM98 marking the end of the linkage map, but the significance of and basis for the apparent split signals remain unknown. In Figure 4, we show examples of the different types of patterns observed, from one locus (Figure 4a) to two loci (Figure 4b,c). In almost every case, we observed signals just proximal to a small chromosomal staining gap that in the paint image. We speculated that the sCBM9.8 BAC contains subtelomeric repeat sequences that are conserved in sequence and position near the end of the chromosome and therefore contribute to the most distal FISH signals observed. To test one prediction of this idea, we labeled total sorghum DNA as a probe, and found that it resulted in a discrete doublet near the end of the

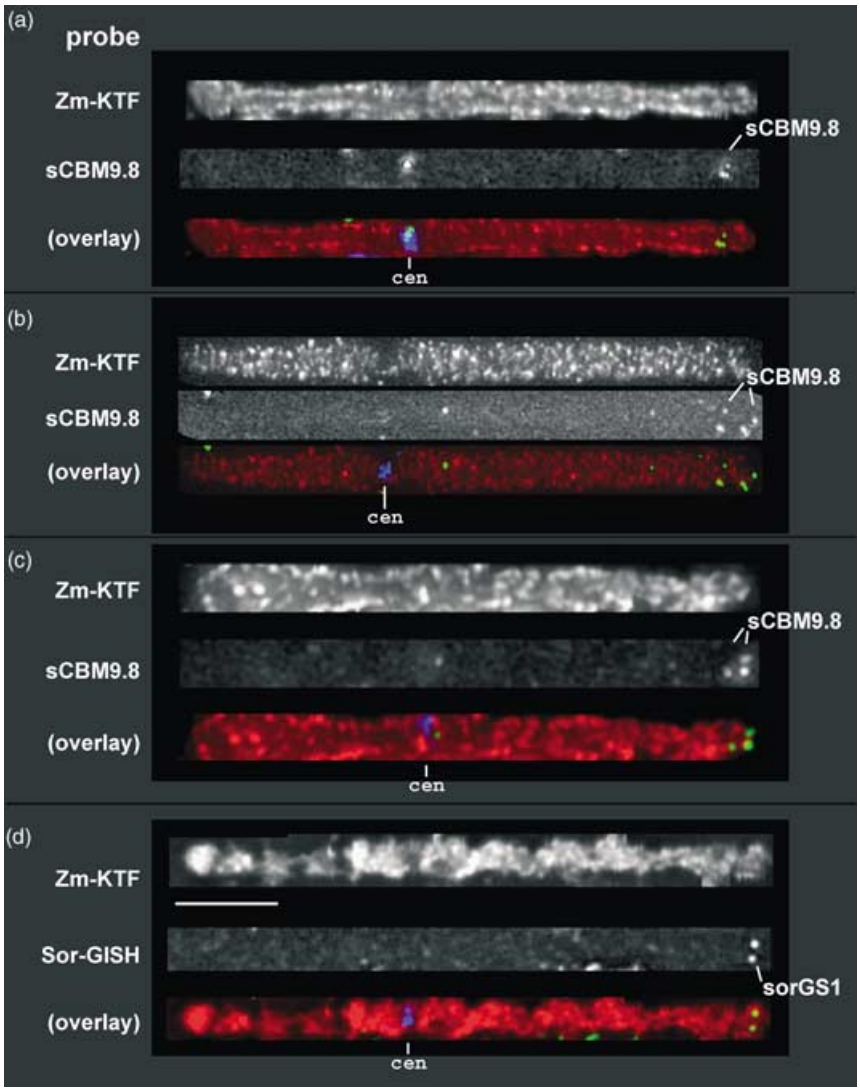

Figure 4. Localization of FISH signals from sCBM9.8.
Chromosomes from spreads were stained, imaged, straightened, and displayed as described for Figure 3. Images are presented as single-wavelength projections (Zm-KTF, sCBM9.8, and Sor-GISH) or as combined images from multiple wavelengths (overlay). (a–c) Three examples illustrate the various patterns of spots observed from the sCBM9.8 probe. Chromosome paint images (Zm-KTF, FITC) are shown above the FISH images (sCBM9.8, rhodamine). The third image in each panel is the color overlay showing the paint (red), sCBM9.8 (green), and centromere (blue, labeled cen). (d) FISH signals at 9L are also observed when total sorghum DNA is used as a painting probe. The bright discrete sorghum GISH signals are referred to as 'sorGS1', and are located in a region that overlaps with the area stained by FISH with the sCBM9.8 probe. Scale bars are 5 μm.

chromosome (Figure 4d). In addition, we tested whether the sCBM9.8 BAC contained sequences that were abundant in the sorghum genome. The restriction-digested sCBM9.8 was subjected to Southern blot analysis with sorghum total DNA as a probe, but we did not detect any particular fragments with higher-than-expected signals (not shown). Therefore, we concluded that sCBM9.8 hybridizes to two loci, one at 9L.95 (±0.03) and the other at 9L.97 (±0.01). The sorghum total DNA GISH probe signals, named SorGS1, mapped to position approximately 9L.975.

sCBM9.1 FISH signals map to 9S.79

For a third locus, we carried out FISH experiments with sCBM9.1 as described above, but with a smaller sample size. Representative images are shown in Figure 5. The sCBM9.1 probe resulted in the appearance of discrete doublets (Figure 5d–f, white arrows). This probe gave a relatively high background of speckles on the chromosome, but the most abundant signals and all of the doublet signals were always near position 9S.79. The discontinuity in staining of the whole fiber (Figure 5b) is thought to have resulted from an overlapping fiber from the oat genome. We frequently saw such gaps and could usually identify a similar criss-cross by inspection of the DAPI image. The sCBM9.1 FISH signals were localized to 9S.79.

Discussion

Single-locus cytogenetic mapping by FISH

The potential value of cytogenetic mapping in maize has been recognized for years, but despite limited success in recent years, we still do not know where most of the genes are located along the pachytene chromosome. Gaining such knowledge would add value to the large collection of chromosomal rearrangement stocks that are available

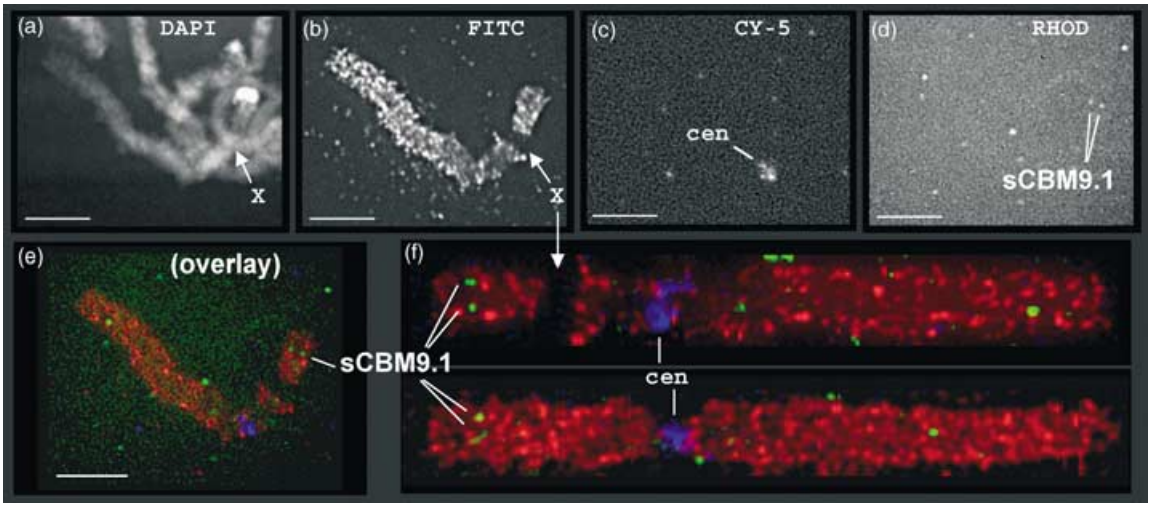

Figure 5. Localization of FISH signals from sCBM9.1. Chromosomes from spreads were stained, imaged, straightened, and displayed as described for Figure 3. Images are presented as single-wavelength projections (a–d) or as combined images from multiple wavelengths (overlay).
(a) The DAPI image shows spread pachytene fibers from a region near the edge of one nucleus. An area of chromosome-fiber criss-cross is indicated (x).
(b) The FITC image shows maize 9 paint from Alexa488-dUTP-labeled maize DNA.
(c) The Cy5 image shows centromere FISH signals (cen).
(d) The rhodamine image shows indirect immunofluorescence signals (sCBM9.1) from digoxigenin-labeled sCBM9.1 BAC DNA.
(e) Color overlays of the FITC (red), Cy-5 (blue), and rhodamine (green) images reveal the position of the centromere (cen) and sCBM9.1 signals on maize 9.
(f) Multiple-wavelength projections of straightened chromosomes (as described for Figure 3f) illustrate the double-dot FISH signals (sCBM9.1) on 9S. Scale bars are 5 μm.

for research, but are underutilized. Weber and colleagues have successfully localized two single-copy loci (*umc105* and *csu45*) onto maize pachytene chromosomes (Sadder and Weber, 2002; Sadder *et al.*, 2000). Their approach involves the use of probes from maize genomic cosmid clones in conjunction with suppression *in situ* hybridization (CSSH) to suppress cross-hybridization of repeat-sequence family segments that are present in the cosmid clone and distributed across other regions of the maize genome. Using ^{3}H-labeled radioactive probes, Shen *et al.* (1987) were able to map the *Waxy1* gene to 9S.02, yet this approach has not been generally adapted to other loci. Here we have essentially doubled the number of loci mapped by FISH to maize pachytene chromosomes. We, therefore, believe that this approach holds great potential for general extension to the development of a high-density cytogenetic map of maize.

A current, united cytogenetic map of maize chromosome 9

Figure 6 shows a cytogenetic map of maize chromosome 9 in which the three new loci from the present study (sCBM9.1, sCBM9.2, and sCBM9.8) are added to those previously mapped to produce a new combined map. Loci from the cytogenetic map have positions based on the fractional-length scheme and are connected to the corresponding loci on the *UMC98* map (Davis *et al.*, 1999). The data generally agree on the order of loci, and their distribution appears to reflect the widely documented discrepancy between linkage and actual distances (discussed by Harper and Cande, 2000). The genetic distance between *umc105a* and *bz1* is 24 cM, 16% of the total 150.4 cM for maize chromosome 9, but the cytological distance is only 4% of the total for this same pair of loci, on the basis of the values in the combined map (Figure 6). Remarkably, an adjacent segment reveals a major difference in the opposite direction. The *umc105a* locus is linked to *wx1*, and the distance between them accounts for 6% of the linkage distance but 19% of the chromosomal data. These large variations suggest that global estimates of distance ratios between different maps may not hold much predictive value for specific individual areas, although all maps should be co-linear. Therefore, development of a high-density cytogenetic map should significantly enhance our comprehension of the irregular distribution of genes along the maize chromosomes.

We used digoxigenin-labeled sorghum BAC DNA probes, but it would also be advantageous to use biotin-labeled probes to allow for two-color simultaneous FISH mapping. Such experiments would address the fine-resolution mapping limitations of our approach. The sCBM9.2 probe corresponds to the maize gene *Bz1*, and we have now mapped it to the cytological position 9S.65. The translocation T4-9_{6222} maps to position 9S.68 on the cytogenetic map (Coe, 1994) and to the *sh1* locus on the genetic map, about 3 cM distal to *bz1* (Rakha and Robertson, 1970). Although more than 30 years separate our determination from that of

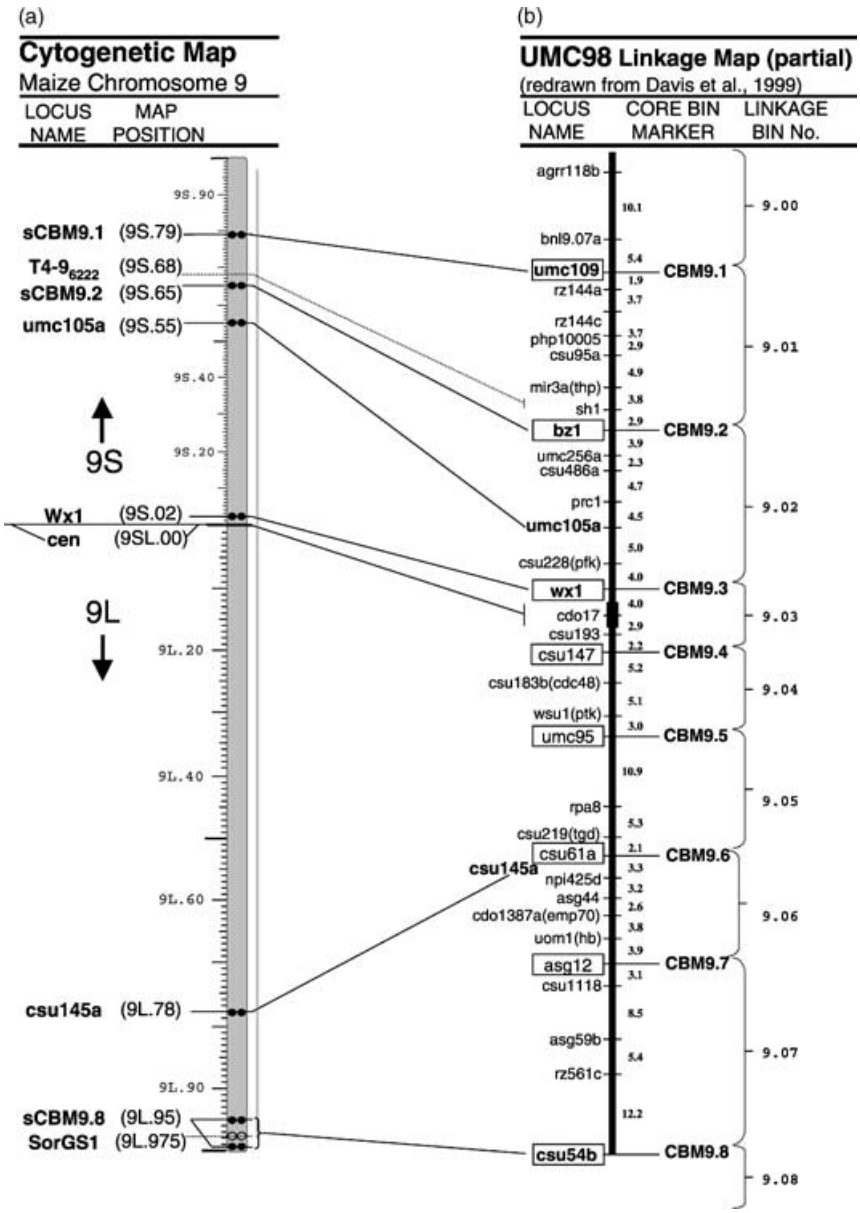

Figure 6. A cytogenetic map for maize chromosome 9 combined with a linkage map. Integration of the cytogenetic and genetic linkage maps for the maize chromosome 9 show the non-uniform relationship between distance (cM) and cytological chromosomal axis distance.
(a) A combined cytogenetic map of maize chromosome 9 showing the positions of eight loci (listed under 'locus' and 'position') that have been mapped to chromosome 9 by *in situ* hybridization (sCBM9.1/umc109, sCBM9.2/bz1, sCBM9.8/csu54b, MCCY/cenC, and sorGS1 from this study; umc105a and csu145a from Sadder and Weber (2001); Wx1/CBM9.3 from Shen *et al.* (1987). The position corresponds to the average relative position along each arm; the centromere is at 0 and the telomeres at 1.0. The positions of T4–9$_{6222}$ on the cytogenetic (Longley, 1961) and linkage maps (Rakha and Robertson, 1970) indicated.
(b) A linkage map redrawn in part from the UMC98 linkage map (Davis *et al.*, 1999). Lines connecting the cytogenetic to the linkage map show that the maps are co-linear at this level and that most of the recombination giving rise to the distances (cM) is disproportionately localized toward the telomere ends of the chromosome arms.

Rakha and Robertson (1970), the comparison of these results illustrates two important aspects of this work. The first is the constancy of the cytogenetic map. Within the limits of error associated with the inherent variation of the system and techniques, the cytogenetic position is fixed, allowing direct comparison of our data to the large body of data previously obtained by inspection of pachytene chromosomes, including landmark studies by McClintock and others. The second is that closely linked loci can be resolved. In this case, a 3-cM distance is equivalent to a cytogenetic distance covering 3/100 of the short arm. Therefore, this approach is probably capable of generating clear mapping data for 40–60 loci on the maize chromosome 9. Finer scale mapping of linked loci could be accomplished by other techniques such as extended-fiber or interphase FISH mapping (Fransz *et al.*, 1996; Jiang *et al.*, 1996; de Jong *et al.*, 1999).

Implications and potential applications of this cytogenetic mapping strategy

The extraordinary DNA sequence localization data that can be obtained with BACs as FISH probes on chromosomes and nuclei has been clearly demonstrated in rice, sorghum, tomato, *Arabidopsis,* and *Medicago trunculata* (Cheng *et al.*, 2001a,b; Islam-Faridi *et al.*, 2002; Kulikova *et al.*, 2001; Lysak *et al.*, 2001; Zwick *et al.*, 1998). In the study reported here, we took advantage of the features of sorghum BACs to develop a single-locus FISH mapping technology suitable for the maize genome. Because this

approach involves cross-species mapping of sorghum genomic sequences onto maize chromosomes, it not only provides a mechanism for integrating the linkage and cytological maps of maize but also establishes a new dimension to the structural-genomics component of sorghum research. Furthermore, using the same sorghum BACs on sorghum chromosomes could permit the development of a new area of genomic research, comparative cytogenetics, possibly bridging the gap between synteny and karyotype comparisons (Paterson *et al.*, 2000). Support for the potential for cross-species cytogenetic mapping within the grass family has been most clearly established in a study of the *liguleless* linkage group in sorghum (Zwick *et al.*, 1998). In that report, rice RFLP markers were used to select, by cross-hybridization, a set of sorghum BAC clones that were then used as FISH probes on sorghum DNA. In addition, Zwick *et al.* (1998) showed that some of these sorghum BAC clones also gave specific FISH signals on chromosomes from both rice and maize. Therefore, we had reason to expect that the general approach would work for maize RFLP-selected sorghum BACs.

In a more general sense, the strategy of using large segments of genomic DNA from small-genome relatives as FISH probes could be used in other plant species for which FISH mapping is complicated by large and complex genomes (Bennett and Smith, 1991; Hanson *et al.*, 2003; Obermayer *et al.*, 2002). Gridded-filter-set BAC libraries are available (http://www.genome.clemson.edu/orders) for a variety of model plant species that have relatively small genomes and may be suitable sources for cross-species BAC FISH probes (Okagaki *et al.*, 2001; Peterson *et al.*, 1999). Small-genome organisms with BAC filter sets that could be used in this way include *Arabidopsis thaliana* (150 Mbp genome size) for some crucifers, *Cucumis melo* (500 Mbp) for some cucurbits, *Lycopersicon esculentum* (950 Mbp) for some Solanaceae, *Liriodendron tulipifera* (790 Mbp) for some Magnoliaceae, *Manihot esculenta* (760 Mbp) for some Euphorbiaceae, *M. truncatula* (525 Mbp) for some legumes, and *Sorghum bicolor* (750 Mbp), *S. propinquum* (750 Mbp), or *Oryza sativa* (430 Mbp) for some Poaceae.

In summary, we have developed and tested a novel strategy for cytogenetic mapping in maize that was made possible by recent genetic and genomic developments in maize, sorghum, and oats. We believe that in principle, and by example, this approach has great potential to solve the old riddle regarding the location and distribution of maize genes along the naturally extended pachytene chromosome fiber. This technology could be combined with other genomics efforts to further enhance the value and power of maize as a model genetic system for basic and applied research, while also revealing a general approach for overcoming FISH-based detection limits for cytogenetics in other species.

Experimental procedures

Plant material and growth conditions

All plant material used was from a disomic maize chromosome 9 addition line of oat (*Avena sativa*) as described by Bass *et al.* (2000b) as oat–maize9b and subsequently designated OMAd9.2 ($2n = 6x = 42 + 2$) by Kynast *et al.* (2001). The commercial sweet corn Seneca 60 was the pollen parent source for the maize chromosome 9 in the original wide-hybrid cross that led to OMAd9.2 (Riera-Lizarazu *et al.*, 1996). Plants were grown at the Florida State University Mission Road greenhouses (Tallahassee, FL, USA), and meiotic stage florets or anthers were fixed within hours of harvest.

3D acrylamide FISH

Anthers from greenhouse-grown plants were fixed in complete meiocyte buffer A (MBA), plus 4% paraformaldehyde for 30 min at room temperature with mild shaking, as previously described (Bass *et al.*, 1997). The fixed anthers were used immediately or stored in MBA at 4°C for up to 6 months before use. On the day of slide preparation, fixed anthers were microdisected, and isolated pollen mother cells were embedded in acrylamide as previously described (Bass *et al.*, 1997); cells from three to four anthers per slide were used. Slides were hybridized, DAPI stained, and imaged as previously described (Bass *et al.*, 2000b), except that the probe mix contained two FISH probes, the maize GISH paint prepared from Knobless Tama Flint (KTF) total DNA and the centromere-specific oligonucleotide probe MCCY. MCCY is 5′-Cy5-GAAAAAC-GAAGAAATGGTTCTGGTG-3′ and was designed to detect the CentC repeat (Ananiev *et al.*, 1998) by hybridizing to the junction between two CentC repeats.

Maize RFLP-based identification of sorghum BAC clones

Sorghum propinquum genomic BAC grid-library filter arrays (filters YRL pair set 01–176, 02–176, 01–177, 02–177) were obtained from A. H. Paterson (University of Georgia, Athens, GA, USA). The filters were screened by hybridization with radiolabeled inserts from plasmids carrying the maize chromosome 9 Core-bin-markers (http://www.agron.missouri.edu/UMCCoreMarkers.html). Probe labeling and hybridization were carried out as previously described (Bass *et al.*, 1994) with 1× SSC-containing aqueous buffers at 68°C, estimated to be at a stringency of approximately T_m: −12°C. The cross-hybridizing BAC clones were obtained, renamed as, for example, 'sCBM9.1' for 'sorghum' BAC with 'CBM9.1'-cross-hybridizing sequences (see Table 2). BAC DNA was isolated with the Qiagen plasmid midi spin kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. BAC DNA was digested with restriction enzymes and was subjected to Southern blot analysis under the same hybridization conditions that are used for initial screening to verify the identity of the clones.

Meiotic chromosome spreads

A meiotic spreading technique was developed by a combination and slight modifications of steps from other protocols based on procedures for cytogenetic analysis of maize pollen mother cells (Dempsey, 1994), mammalian chromosome spreading (Henegariu *et al.*, 2001), and FISH mapping onto tomato pachytene chromosomes (Zhong *et al.*, 1996). Specifically, meiosis-stage florets were harvested and fixed in ethanol:glacial acetic acid (3 : 1), then

stored at −20°C. Pachytene chromosomes were spread by the methods of Zhong *et al.* (1996) with the following modifications designed to incorporate methods from Henegariu *et al.* (2001). After the partial evaporation of the added fixative, the slides were exposed to 75°C water vapor for 3 sec; then four to six drops of glacial acetic acid were added, followed by another exposure to 75°C water vapor for 5 sec. The slides were then dried quickly on a metal heat block at 75–90°C depending on the degree of the desired spreading as previously described (Henegariu *et al.*, 2001). We inspected spreads under phase-contrast microscopy to choose the slides with the best combination of spreading and pachytene chromosome content.

Probe preparation for FISH with chromosome spreads

The maize chromosome 9 and the centromere probes were the same as described above for the 3D acrylamide FISH. For each sorghum BAC (sCBM9.1, sCBM9.2, and sCBM9.8), DNA was isolated by means of the Qiagen midi-plasmid prep kit (Qiagen) and labeled with a digoxigenin High Prime labeling kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. Labeled probes were stored at −20°C until used.

Slide pretreatment and FISH with chromosome spreads

Pachytene chromosome spreads were treated with RNaseA and pepsin according to the method of Zhong *et al.* (1996), then fixed with 2% paraformaldehyde (EM Sciences, Fort Washington, PA, USA) in 1× PBS pH 7.0 for 10 min, washed in 2× standard saline citrate (SSC) for 5 min, denatured in 70% formamide (Sigma, St Louis, MO, USA) and 2× SSC for 5 min at 70°C on a metal heat block, and dehydrated in an ethanol series consisting of ice-cold 70% EtOH, room temperature (RT) 90% EtOH, and RT 100% EtOH for 2 min each. The FISH probes were mixed in a hybridization buffer of 50% formamide and 2× SSC with the maize paint at 9 μg ml^{-1}, MCCY at 4 μg ml^{-1}, and digoxigenin-labeled BACs at 1–5 μg ml^{-1} plus heterologous, denatured unlabeled DNA (calf thymus) at 100 μg ml^{-1}. In some experiments, excess unlabeled sorghum total DNA was included at 40 μg ml^{-1}, which seemed to decrease the number of background spots on and around the chromosomes.

For hybridizations, the probe mix was denatured in 50% formamide and 2× SSC at 90°C for 5 min. The slides carrying the denatured chromosomes were then warmed at 42°C for 5 min, and the probe mix was applied and sealed with a 22 mm × 30 mm cover slip and rubber cement. Hybridization was allowed to proceed for 16 h at 37°C and followed by the following series of washes: 4× SSC and 50% formamide, 30°C, 5 min; 2× SSC, RT, 5 min; and 1× PBS, RT, 5 min. Next, the slides were prepared for immunodetection of the digoxigenin according to the Fluorescent Antibody Enhancer Set for DIG Detection manual (Roche Molecular Biochemicals). The chromosomes were counterstained with DAPI, washed twice with 1× PBS plus 1 mM dithiothreitol, air dried at RT in the dark, mounted in Vectashield (Vector Laboratories, Burlington, CA, USA), sealed with nail polish, and viewed immediately or stored at −80°C.

3D deconvolution microscopy and image analysis

We imaged the chromosomes as previously described (Bass *et al.*, 2000a) to obtain multiple-wavelength 3D optical reconstructions, which were de-blurred by 3D iterative deconvolution and cropped for analysis and display. The path of the chromosome fiber was computationally traced and straightened by means of the Priism software program 'STRAIGHT' (API Softworks and IVE version 3.2). A cylinder about the hand-modeled path of the chromosome is displayed as straightened through-focus projections, preserving the real space positions of signal along the length of the chromosome, allowing actual and relative distance measurements to be made. Cytogenetic coordinates for maize are based on the fractional distance along a given arm, where 9S.33 would represent a locus that is 33% of the distance down the chromosome 9 short arm. The telomere is, by definition, at position 1.0 and the centromere at 0.0.

Acknowledgements

We thank Ruth D. Swofford for excellent technical assistance with 3D image analysis. We thank A. H. Paterson and J. E. Bowers for advice and assistance throughout the project. We thank Bob Getts and Genisphere, Inc., for their generosity and support of experimental reagent development. We thank Lloyd M. Epstein and Ann Morris for critical reading and helpful comments on the manuscript. This work was supported by a graduate fellowship to G.L.K. from The Consortium for Plant Biotechnology Research, Inc., and by a Program Enhancement Grant to H.W.B. from The Florida State University Research Foundation.

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