RESEARCH ARTICLES

High-Resolution Single-Copy Gene Fluorescence in Situ Hybridization and Its Use in the Construction of a Cytogenetic Map of Maize Chromosome 9

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High-resolution cytogenetic maps provide important biological information on genome organization and function, as they correlate genetic distance with cytological structures, and are an invaluable complement to physical sequence data. The most direct way to generate a cytogenetic map is to localize genetically mapped genes onto chromosomes by fluorescence in situ hybridization (FISH). Detection of single-copy genes on plant chromosomes has been difficult. In this study, we developed a squash FISH procedure allowing successful detection of single-copy genes on maize (Zea mays) pachytene chromosomes. Using this method, the shortest probe that can be detected is 3.1 kb, and two sequences separated by \(-100\) kb can be resolved. To show the robust nature of this protocol, we localized nine genetically mapped single-copy genes on chromosome 9 in one FISH experiment. Integration of existing information from genetic maps and the BAC contig-based physical map with the cytological structure of chromosome 9 provides a comprehensive cross-referenced cytogenetic map and shows the dramatic reduction of recombination in the pericentromeric heterochromatic region. To establish a feasible mapping system for maize, we also developed a probe cocktail for unambiguous identification of the 10 maize pachytene chromosomes. These results provide a starting point toward constructing a high-resolution integrated cytogenetic map of maize.

INTRODUCTION

Maize (Zea mays) is one of the most important crops in the world and has long been a model species for research. A comprehensive understanding of the maize genome serves as the foundation for numerous studies. Since the launch of a plant genome initiative by the U.S. National Science Foundation in 1998 (Pennisi, 1998), a large number of resources for analysis of the maize genome have become available. The IBM map, a high-density genetic map for the intermated B73/Mo17 population containing \(1850\) markers, was constructed (Lee et al., 2002; Sharopova et al., 2002). BACs were fingerprinted and assembled into a fingerprint contig map (Soderlund et al., 2000), and 721 contigs covering \(93\%\) of the maize genome have been generated (http://www.genome.arizona.edu/fpc/maize/), and 85\% of the contigs were anchored to the genetic maps (http://www.maizemap.org/). These resources are extremely valuable and will provide strong support for sequencing of the maize genome. However, even when the sequences of the entire genome are available, this large amount of genetic and physical sequence data cannot be directly related to chromosome structure.

Cytogenetic maps show the positions of genetically mapped markers on chromosomes, relative to cytological landmarks such as centromeres, telomeres, heterochromatin, and nucleolar organizer regions. With the advance of genome research, cytogenetic maps will not only be valuable for integrating and organizing genetic, molecular, and cytological information, but they will also provide a unique insight into genome organization in the context of the chromosomes. In plants, two general approaches have been adopted for construction of a cytogenetic map (for reviews, see Harper and Cande, 2000; Wang and Chen, 2005). The first general strategy, widely used in wheat (Triticum aestivum), barley (Hordeum vulgare), tomato (Lycopersicon esculentum), maize, and others, is to locate genetically mapped DNA markers to chromosomal segments relative to cytologically mapped breakpoints using cytogenetic stocks (McClellan, 1944; Khush and Rick, 1968; Weber and Helentjaris, 1989; Beckett, 1991; Werner et al., 1992; Coe et al., 1993; Lin et al., 1997; Kunzel et al., 2000). The second strategy is to directly visualize DNA sequences on chromosomes by fluorescence in situ hybridization (FISH). This technique has been widely used for cytogenetic and genome research, mainly for mapping large genomic clones and repetitive sequences. Localization of large genomic clones, such as bacterial or yeast artificial chromosomes, on chromosomes by FISH and integration of cytological and genetic maps have been demonstrated in several plants (Zhong et al., 1999; Fransz et al., 2000; Cheng et al., 2001a; Kulikova et al., 2001; Howell et al., 2002; Islam-Faridi et al., 2002; Koumaris and Bass, 2003). The importance of chromosomal mapping of DNA by FISH to the genome research efforts have
been distinctly illustrated by the molecular and cytological characterization of a heterochromatic knob on the short arm of Arabidopsis thaliana chromosome 4 (Franz et al., 2000) and the confirmation of anchored BAC contigs and gene orders in rice (Oryza sativa) (Zhao et al., 2002). In maize, however, the use of BAC clones as FISH probes has been very difficult because maize BAC clones contain large amounts of repetitive elements and their use as FISH probes results in a cross-hybridization throughout the genome. However, one group has detected two maize cosmid clones on maize chromosomes by adding Cot-100 DNA to suppress the repetitive sequences presented in the probes (Sadder et al., 2000; Sadder and Weber, 2002). Recently, Koumbaris and Bass (2003) have developed a strategy to avoid the cross-hybridization of repetitive sequences using sorghum (Sorghum bicolor) BACs as probes to the maize chromosome 9 in an oat (Avena sativa)-maize addition line.

The most direct and effective way to construct cytogenetic maps for organisms with large and complex genomes like maize is to directly localize single-copy genes on chromosomes by FISH. However, the detection of small unique sequences on plant chromosomes has been difficult because the debris of cell wall and cytoplasm reduces the accessibility of target DNA and increases background and consequently results in a relatively low signal-to-noise ratio (Lehfe et al., 1993; Jiang et al., 1995). Nevertheless, successful detection of single-copy genes has been reported by several laboratories. For example, Franz et al. (1996) mapped the chalcone synthase A gene on Petunia hybrida mitotic metaphase chromosomes with a 1.4-kb probe, and Ohmido et al. (1998) localized a 1.29-kb sequence on rice mitotic metaphase chromosomes; these results are comparable to similar work on the human genome (Min and Swansbury, 2003). Detection of probes as small as 4 kb on maize interphase nuclei was also reported (Jiang et al., 1996). Another approach to increasing FISH sensitivity is to use tyramide-FISH, in which signals can be amplified by the enzymatic deposition of fluorochrome-conjugated tyramide, and target sequences as small as 710 bp on Allium cepa mitotic chromosomes could be detected (Khrustaleva and Kik, 2001). However, these reports involved mapping small unique sequences on mitotic metaphase chromosomes. Using metaphase chromosomes as a target for FISH limits mapping resolution and the visibility of chromosome-associated cytological structures.

Meiotic pachytene chromosomes have been shown to be a better target for high-resolution FISH mapping in plants (Xu and Earle, 1996; Zhong et al., 1996a, 1996b; Franz et al., 1998; Chen et al., 2000). First, pachytene chromosomes are generally 10 to 20 times longer than somatic metaphase chromosomes and possess many cytological landmarks (de Jong et al., 1999; Chen et al., 2000; see Supplemental Table 1 online). Second, the wall of pollen mother cells can be easily digested by enzyme treatment, thus facilitating probe penetration. Third, four copies of the DNA sequences of interest are present on pachytene bivalents, so signals will be stronger on pachytene chromosomes than on somatic metaphase chromosomes. Finally, the pachytene stage is biologically relevant to genetic maps since pachytene is thought to be the stage when recombination is completed (Roeder, 1997), and recombination occurs at a much higher frequency within genes than outside genes (Civardi et al., 1994; Fu et al., 2002). An accurate picture of where the genes are during meiotic recombination could provide novel insights into meiotic processes.

In a previous study, we found that three-dimensional (3-D) FISH, which is known to preserve the nuclear architecture and chromatin morphology, is not suitable for single-copy gene mapping on maize pachytene chromosomes. Chen et al. (2000) demonstrated that a mild squash procedure produced high-quality pachytene chromosomes suitable for high-resolution mapping with repetitive sequence probes. Here, we describe a set of probes for unambiguous identification of the 10 maize pachytene chromosomes and compare the karyotypes derived from the squash and 3-D chromosome preparations to evaluate the preservation of chromosome morphology after squash procedure. To detect small unique sequences, we optimize the mild squash FISH protocol and show that single-copy gene probes as small as 3.1 kb can be accurately and reproducibly mapped on pachytene chromosomes and that two closely linked single-copy genes separated by ~100 kb can be resolved. To demonstrate the utility of this technique for genomic studies, we show that a cocktail of nine single-copy probes can detect nine loci on pachytene chromosome 9 in a single experiment and correlate these FISH mapping data with existing information from genetic and physical maps to generate an integrated cytogenetic map of maize chromosome 9. Finally, we illustrate two applications of this technique.

RESULTS

Unambiguous Identification of Pachytene Chromosomes with a Marker Cocktail

Chromosome identification is of prime importance for the construction of cytogenetic maps. Although the maize pachytene chromosomes have been widely used for cytogenetics, recognition of each chromosome has relied on the measurement of chromosome length, arm ratio, presence of specific amounts of heterochromatin and knobs, and the experience of the cytologist (Dempsey, 1994). Mitotic metaphase chromosomes are much harder to distinguish than meiotic pachytene chromosomes, and Kato et al. (2004) have developed an excellent set of probes with nine repetitive sequences that can be used to identify each mitotic chromosome.

Since there are more cytological landmarks on pachytene than mitotic chromosomes, we developed a marker cocktail containing six different repetitive sequences for identifying all pachytene chromosomes accurately and rapidly. This cocktail is composed of centromeric repeat (CentO) (Ananiev et al., 1998b), telomere sequences, 5S and 45S rRNA genes, and the 180-bp (Peacock et al., 1981) and TR-1 (Ananiev et al., 1998a) knob repeated sequences. These repetitive sequences have been localized on maize pachytene chromosomes in separate experiments (Chen et al., 2000; Hsu et al., 2003; Wang and Chen, 2003). To place all these markers on chromosomes in a single FISH experiment, six different repetitive sequences were labeled with either one or two fluorochromes and were hybridized to pachytene chromosomes of the Kansas Yellow Saline (KYS) inbred line. These six probes showed distinct and consistent foci on pachytene chromosomes (Figure 1A).
Figure 1. Pachytene Chromosome Identification Based on the Distribution of Repetitive DNA Sequences in Maize Inbred Line KYS Using FISH.

In (A) and (B), chromosomes were prepared by the squash procedure, and in (C) and (D), meiocytes were mounted in an acrylamide pad to preserve the 3-D chromosome structure. The 4′,6-diamidino-2-phenylindole (DAPI)–stained chromosomes were converted to black-and-white images and overlaid with signal images. Six repetitive sequences labeled with different fluorochrome combinations show distinct colors on pachytene chromosomes as follows: centromeric CentC repeat (red), telomere sequences (dark blue), 180-bp repeat (green), TR-1 element (pink), and 5S (light blue) and 45S (yellow) rRNA genes. In (A), (B), and (D), all signals are indicated by arrowheads of the same color corresponding with signals except for centromeres and telomeres. TR-1 elements coexisting with 180-bp repeats in the terminal knob on 6S and the tip of 6L are white and are indicated by white arrowheads. The small knob on 4S is indicated by a small white arrow. Bars = 10 μm.

(A) Pachytene chromosomes derived from squash preparations were probed with the marker cocktail. The presence of two sites of CentC signals on chromosome 10 is indicated by a red arrow, and the isolated centromere 10 is shown in the inset.

(B) Computationally straightened chromosomes were taken from the cell shown in (A). Large white arrow indicates centromeres. Note that small lines almost perpendicular to the straightened chromosome are traces of other chromosomes that cross the straightened chromosome. The inset shows only the telomere signal on 9S.

(C) A projection after deconvolution of a 3-D pachytene meiocyte shows signals of the marker cocktail probes.

(D) Computationally straightened chromosomes were taken from the cell shown in (C). Some small loci were not detected in 3-D preparations. White arrow indicates centromeres.
Based on the distribution of these repetitive sequences, 10 pachytene bivalents of KYS can be unambiguously identified. Computationally straightened chromosomes taken from the cell in Figure 1A are shown in Figure 1B. The positions of these markers were consistent with the observations in the previous studies (Chen et al., 2000; Hsu et al., 2003; Wang and Chen, 2003); however, using this marker cocktail allowed unambiguous identification of all pachytene chromosomes in one FISH experiment. Ten centromeres were marked by the CentC repeats, but the hybridization signals on chromosomes 1, 2, 6, 7, 9, and 10 were usually stronger than those on the remaining chromosomes. Interestingly, there were two distinct sites of CentC signals on chromosome 10; one was brighter than the other (see the inset in Figure 1A showing a magnified region of chromosome 10). A probe against the CentA element, another centromeric DNA sequence (Ananiev et al., 1998b), colocalized with all CentC signals on centromeres, including the two loci of chromosome 10 (see Supplemental Figure 1 online). It is not known whether either one or both loci contribute to centromere function. The telomere signals were found at the termini of all chromosome arms except the short arm of chromosome 9 (9S), where telomere signals were usually observed proximal to the terminal knob (see the inset in Figure 1B showing a magnified region of chromosome 9 and Figures 2C, 2E, and 2G). It seems unlikely that telomere

![Figure 2. Mapping of bz1, d3, and wx1 Genes on Pachytene Chromosomes Using HR Gene FISH.](image)

(A) to (C) Pachytene chromosomes of maize inbred line KYS were probed with 13.3-kb bz1 fragment (red) and FITC-labeled CentC repeats and telomere sequences (green). Note the presence of two CentC signals on chromosome 10 indicated by a white arrow. (B) shows a magnified image of chromosome 9, and the computationally straightened chromosome is shown in (C).

(D) and (E) Detection of 3.4-kb d3 fragment (red) on pachytene chromosome 9. The computationally straightened chromosome 9 taken from (D) is shown in (E).

(F) and (G) Detection of a 3.1-kb wx1 fragment (red) on pachytene chromosome 9. The computationally straightened chromosome 9 taken from (F) is shown in (G).

The three straightened chromosomes in (C), (E), and (G) are shown at the same magnification. The asterisks indicate the terminal knob on 9S. Bars = 10 μm.
Comparison of Karyotypes Derived from Squash and 3-D Chromosome Preparations

In cytogenetic maps, the chromosomal positions of markers are usually reported as a fraction of total chromosome arm length, so the accuracy and consistency of mapping depends on preservation of chromosome morphology. To ensure that the chromosomes derived from the squash preparation maintain their relative lengths and reflect relevant positions, we compared the karyotypes derived from the squash and the 3-D chromosome preparations using the marker cocktail as probes. The 3-D chromosome preparation has been shown to preserve the chromosome morphology (Belmont et al., 1987; Bass et al., 1997). An example of a projection image of a 3-D nucleus subjected to FISH with the marker cocktail is shown in Figure 1C, and 10 straightened chromosomes isolated by computer-aided software from the same cell are lined up in Figure 1D (same magnification as Figure 1B). The hybridization pattern of the marker cocktail also allowed all chromosomes in 3-D–prepared meiocytes to be identified, although it failed to detect some small signals because of the lower probe accessibility and hybridization efficiency.

Fourteen and five pachytene-stage meiocytes derived from the squash and 3-D preparations, respectively, were selected for measurements of the karyotype. The absolute chromosome lengths in micrometers, the percentages of total complement length, and arm ratios were calculated (Table 1). The means of total length of the pachytene bivalents from squash and 3-D preparations are 724.69 and 418.81 μm, respectively (i.e., they differ by a factor of 1.7). However, as shown in Table 1, the arm ratios of each chromosome obtained from both methods are consistent and similar with those obtained from the classic acetocarmine staining method, suggesting that our squash chromosome preparation produces longer chromosomes than that observed in 3-D fixed cells but leaves arm ratios of the chromosomes unaltered.

To determine whether relative distances within the chromosomal arm were consistent from one method to the other and to evaluate the accuracy of mapping results generated from the squash preparation, we compared the relative positions of foci on squash and 3-D–prepared chromosomes subjected to FISH with the marker cocktail. The positions of 5S rDNA on 2L, TR-1 elements on 4L, and knobs on 5L, 6L, and 7L derived from both methods were determined (Table 2). We also mapped the 22-kD α-zein gene cluster, which is present on the short arm of chromosome 4 (see Supplemental Figure 2 online) as a cluster of ∼23 genes spanning ∼160 kb physically (Llaca and Messing, 1998; Song et al., 2001). The relative positions of all six markers obtained by both methods were consistent with each other (Table 2). This result indicates that relative positions of genes on pachytene chromosomes after the squash preparation are preserved.

### Table 1. Chromosome Lengths and Arm Ratios of Maize Pachytene Chromosomes Derived from the Two Methods Used in This Study and the Classic Acetocarmine Squash Technique

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Length (μm)</td>
<td>TCL (%)</td>
<td>L:S</td>
<td>Length (μm)</td>
</tr>
<tr>
<td>1</td>
<td>108.79 ± 11.51</td>
<td>15.03 ± 0.64</td>
<td>1.30 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>86.78 ± 10.24</td>
<td>12.04 ± 0.62</td>
<td>1.14 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>82.55 ± 8.32</td>
<td>11.39 ± 0.49</td>
<td>2.02 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>76.50 ± 6.91</td>
<td>10.58 ± 0.76</td>
<td>1.58 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>80.21 ± 7.21</td>
<td>11.08 ± 0.59</td>
<td>1.97 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>60.30 ± 6.03</td>
<td>8.32 ± 0.41</td>
<td>3.49 ± 0.41</td>
</tr>
<tr>
<td>7</td>
<td>62.64 ± 6.33</td>
<td>8.63 ± 0.39</td>
<td>2.70 ± 0.14</td>
</tr>
<tr>
<td>8</td>
<td>61.23 ± 7.55</td>
<td>8.44 ± 0.57</td>
<td>3.16 ± 0.21</td>
</tr>
<tr>
<td>9</td>
<td>56.07 ± 7.97</td>
<td>7.73 ± 0.77</td>
<td>1.83 ± 0.15</td>
</tr>
<tr>
<td>10</td>
<td>49.62 ± 5.81</td>
<td>6.85 ± 0.62</td>
<td>2.61 ± 0.13</td>
</tr>
</tbody>
</table>

a Chromosome number.
b TCL, total complement length.
c L:S, ratio of chromosome long arm length to short arm length.
d Calculated by authors of this article.

Dempsey (1994).
The Detection of Unique Gene Sequences on Pachytene Chromosomes

To detect unique gene sequences, the squash FISH procedure was optimized for a maximal signal-to-noise ratio, and we termed it high-resolution single-copy gene FISH (HR gene FISH). As described in Methods, slides prepared by the mild squash procedure were treated with a low concentration of pepsin to digest the debris in the cytoplasm. The incubation time of pepsin digestion was adjusted depending on the previous chromosome preparation steps and the appearance of the chromosomes and varied from 20 to 60 min (see Supplemental Figure 3 online). Chromosomes prepared in this way were hybridized with probes that lacked repetitive DNA and were directly labeled with a fluorochrome, such as Cy3 and fluorescein isothiocyanate (FITC). Subsequent washes were done at a high stringency. These adaptations facilitate probe penetration and reduce background fluorescence.

We labeled a 13.3-kb maize genomic DNA fragment from the clone pHF10 with Cy3 and the CentC and telomere sequences with FITC. The 13.3-kb fragment contains the serine threonine kinase1 (stk1), bronze1 (bz1), and sesquiterpene cyclase1 (stc1) genes and is known as a gene-rich region (Fu et al., 2001), so we treated this probe as a single locus, representing the bz1 gene. In FISH analysis, the 13.3-kb fragment was detected on 9S and showed a bright paired hybridization spot on synapsed homologous chromosomes in all 24 cells observed (Figures 2A to 2C). In KYS, chromosome 9 can be easily identified without using the marker cocktail by the conspicuous knob at the tip of the short arm. In a well-spread pachytene cell, such as the one shown in Figure 2A, all chromosomes can be identified based on arm ratio and the intensity of CentC signals. For determining the accurate position of probes, 18 chromosomes without apparent morphological distortion were computationally straightened (e.g., Figure 2C), and the bz1 locus was determined at a position of 72.32% of the distance from the centromere to the telomere on 9S; that is cytogenetic position 9S.72.

Table 2. Locations of Markers on Pachytene Chromosomes Derived from Two Different FISH Methods

<table>
<thead>
<tr>
<th></th>
<th>5S rDNA on 2L</th>
<th>n</th>
<th>Zein on 4S</th>
<th>n</th>
<th>TR-1 on 4L</th>
<th>n</th>
<th>Knob on 5L</th>
<th>n</th>
<th>Knob on 6L</th>
<th>n</th>
<th>Knob on 7L</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squash method</td>
<td>83.87 $\pm$ 1.07</td>
<td>14</td>
<td>91.59 $\pm$ 1.91</td>
<td>11</td>
<td>89.01 $\pm$ 1.44</td>
<td>14</td>
<td>72.81 $\pm$ 2.60</td>
<td>14</td>
<td>76.46 $\pm$ 2.16</td>
<td>14</td>
<td>66.29 $\pm$ 2.99</td>
<td>14</td>
</tr>
<tr>
<td>3-D method</td>
<td>84.50 $\pm$ 0.98</td>
<td>8</td>
<td>91.72 $\pm$ 1.43</td>
<td>4</td>
<td>88.56 $\pm$ 0.98</td>
<td>4</td>
<td>73.42 $\pm$ 1.45</td>
<td>6</td>
<td>77.26 $\pm$ 1.57</td>
<td>8</td>
<td>68.08 $\pm$ 2.40</td>
<td>9</td>
</tr>
</tbody>
</table>

* The location is given as the percentage distance from centromere to signal site over the chromosome arm.
* The number of chromosomes used for measurements.

Single-Copy Sequences as Small as 3.1 kb Can Be Detected

To test the sensitivity of this method, we cloned several smaller DNA fragments corresponding to the colored aleurone1 (c1), waxy1 (wx1), and dwarf3 (d3) genes based on sequence data available in the database (see Methods and Supplemental Table 2 online). These three genes have been genetically mapped to 33.4, 63.7, and 66 centimorgans (cM), respectively, on chromosome 9 of the Genetic 2005 map (MaizeGDB; http://www.maizegdb.org/). For FISH analysis, single gene fragments were labeled with Cy3 and mixed with centromere and telomere probes and then hybridized with pachytene chromosomes. To determine the lower limit of probe length that can be detected, hybridizations for each probe were repeated in at least two independent experiments, each comprising two slides with high-quality chromosome spreads. We randomly selected cells and captured the image using a high-sensitivity cooled charge-coupled device (CCD) camera. Only hybridization signals that appeared as distinct double spots on the bivalent before deconvolution were considered as labeled.

A 3.4-kb DNA fragment containing the d3 gene was detected in 28 of 40 observed cells (70%) and mapped at position 9S.36 (Figures 2D and 2E). For the wx1 gene, when a 3.1-kb fragment (wx1-5) containing only the gene region was used as probe, the hybridization signals were observed in 19 of the total 30 examined cells (63%) and mapped at position 9S.46 (Figures 2F and 2G). However, another wx1 fragment (wx1-2), which is 4.1 kb in length, showed hybridization signals in only 12 of the total 30 cells (40%) and produced a fuzzy cross-hybridization background along chromosomes (data not shown). This was likely due to the fact that the 5’ end of the wx1-2 fragment carries 311 bp of a Heartbreaker element, which is a member of a very large family of miniature inverted-repeat transposable elements widely dispersed throughout the maize genome (Casa et al., 2000; Zhang et al., 2000). The sensitivity of our technique was further tested using a 1.3-kb fragment of the c7 gene, and no signals were observed on 9S. From these tests, we concluded that single-copy sequences as short as 3.1 kb can be mapped on maize pachytene chromosomes by HR gene FISH.

Mapping Resolution of HR Gene FISH

The resolving power of FISH can be defined as the ability to distinguish two adjacent DNA sequences and determine their order using corresponding probes with different labels. To measure the resolution of our technique, we selected the sh1-to-bz1 interval for analysis. The loci sh1 and bz1 are genetically mapped to 36.4 and 39.3 cM on 9S and present on the same BAC contig (ctg371; http://www.genome.arizona.edu/fpc/WebAGCoL/maize/WebChrom/wcmaize9.htm). The physical distance between these two genes is ~250 kb in B73 (F. Wei, personal communication). In FISH experiments, we simultaneously used the FITC-labeled sh1 and Cy3-labeled bz1 (pHF10) probes, which were mixed with Cy3-labeled centromere probes. The FISH signals derived from these two gene probes were separated from each other, and the order of signals on chromosome 9 could be determined in all 15 cells examined (Figures 3A to 3C). The signals derived from pHF10 were always toward the centromere, and the orientation of these two genes was consistent with the genetic data.
The *bz1* region has been characterized in detail (Fu et al., 2001, 2002; Fu and Dooner, 2002). They have sequenced >100 kb from the *bz1* region of two maize lines, McC and B73. The molecular structure they explored is diagrammed in Figure 3D. Based on their data, *ubiquitin conjugating enzyme2* (*uce2*) and *tac7077* are adjacent to each other and are 28 kb from the *stc1-*bz1-*stk1* region in B73 and 93 kb in McC. The genetic distance between *tac7077* and *bz1* is <1 cM (H. Dooner, personal communication). The physical distance between the two regions in KYS hasn’t been reported. We assume it is similar in size to B73 because the pachytene chromosome lengths of KYS and B73 are similar and there is no apparent difference in the distribution of major chromomeres in that region of chromosome 9 in the two inbred lines (K. Dawe, C.-J.R. Wang, and W.Z. Cande, unpublished data). To test whether HR gene FISH can resolve this small physical distance, we labeled *tac7077* (2.5 kb) and *uce2* (1.8 kb) with FITC and the pHF10 clone with Cy3 and then probed to KYS pachytene chromosomes. Figure 3E shows a representative image, and the magnified signals are shown in Figure 3F. The *tac7077* and *uce2* probes labeled with the same fluorochrome resulted in distinct green signals that were adjacent to the red bright signals generated by pHF10 probe. In 13 cells examined, five cells showed partially overlapped signals. The order of signals relative to centromeres on all 13 bivalents was consistent with the molecular data. These tests suggest that two loci separated by ~100 kb can be resolved on pachytene chromosomes by the HR gene FISH.

**Simultaneous FISH Mapping of Multiple Genes on Pachytene Chromosome 9**

To demonstrate how cytogenetic maps can be constructed by HR gene FISH, we cloned *restorer of fertility2* (*rf2*), *glossy15* (*gl15*), *SBP-domain protein4* (*sbp4*), and *sucrose synthase1* (*sus1*) (see Methods and Supplemental Table 2 online), which have been genetically placed on chromosome 9. Together with the single-copy genes mentioned above, nine gene probes were labeled alternately with FITC or Cy3 after their chromosomal order was confirmed in separate FISH experiments (Table 3). The nine single-copy probes represent 12 genes, in which the *tac7077* and *uce2* sequences labeled with FITC represent one locus (referred to here as *tac7077*), and the pHF10 clone contains
showed that the group 7 on the Genetic 1997 map; however, the FISH results marked centromeres and telomeres on chromosome 9. Even the cocktail produced nine alternate red/green signals of genes and chromosome 9 (Figure 4A). Three straightened chromosomes zation of some of the probes, approximately one-fifth of the KYS pachytene chromosomes. While all cells showed hybridization of some of the probes, approximately one-fifth of the observed cells showed the hybridization signals of all probes on chromosome 9 (Figure 4A). Three straightened chromosomes 9 from three meiocytes are lined up in Figure 4B. This probe cocktail produced nine alternate red/green signals of genes and marked centromeres and telomeres on chromosome 9. Even the sh1-tac7077-bz1 region could be resolved as two pairs of red signals derived from sh1 and bz1 probes, and the green signal of tac7077 was present between sh1 and bz1 signals. The yellow fluorescence between the red and green spots represents the region of signal overlap.

Six pachytene chromosomes 9 without morphology distortion showing all hybridization spots were selected for analyzing the locations of signals. We separated the results obtained by the simultaneous FISH mapping from the previous mapping data obtained in the individual FISH experiments and listed the mapping values in Table 3. The locations of each probe obtained from the two sets of FISH experiments were consistent. We combined the data sets to calculate the average positions of the nine probes. This experiment shows that our method is an effective technique for construction of a cytogenetic map.

### Table 3. Physical Locations of Gene Probes on Pachytene Chromosome 9

<table>
<thead>
<tr>
<th>Probes</th>
<th>Chromosomal Arm</th>
<th>Separate FISH Experiments</th>
<th>Nine-Gene Probe Cocktail</th>
<th>n</th>
<th>Total</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh1</td>
<td>9S</td>
<td>75.03 ± 0.53</td>
<td>74.30 ± 1.52</td>
<td>6</td>
<td>74.74 ± 1.06</td>
<td>15</td>
</tr>
<tr>
<td>uce2 and tac7077</td>
<td>9S</td>
<td>73.85 ± 1.63</td>
<td>72.38 ± 1.16</td>
<td>6</td>
<td>73.45 ± 1.71</td>
<td>22</td>
</tr>
<tr>
<td>stc1, bz1, and stk1</td>
<td>9S</td>
<td>72.32 ± 1.96</td>
<td>71.13 ± 1.88</td>
<td>6</td>
<td>72.02 ± 1.98</td>
<td>24</td>
</tr>
<tr>
<td>wx1</td>
<td>9S</td>
<td>45.65 ± 2.74</td>
<td>46.62 ± 1.58</td>
<td>6</td>
<td>45.93 ± 2.46</td>
<td>21</td>
</tr>
<tr>
<td>k3</td>
<td>9S</td>
<td>36.60 ± 2.27</td>
<td>38.43 ± 1.51</td>
<td>6</td>
<td>36.93 ± 2.25</td>
<td>32</td>
</tr>
<tr>
<td>rt2</td>
<td>9S</td>
<td>27.74 ± 1.60</td>
<td>28.57 ± 1.02</td>
<td>6</td>
<td>27.91 ± 1.52</td>
<td>29</td>
</tr>
<tr>
<td>g15</td>
<td>9L</td>
<td>28.58 ± 1.18</td>
<td>27.78 ± 0.72</td>
<td>6</td>
<td>28.28 ± 1.08</td>
<td>16</td>
</tr>
<tr>
<td>sbp4</td>
<td>9L</td>
<td>35.65 ± 0.79</td>
<td>36.46 ± 0.77</td>
<td>6</td>
<td>35.95 ± 0.86</td>
<td>16</td>
</tr>
<tr>
<td>sus1</td>
<td>9L</td>
<td>49.20 ± 1.63</td>
<td>49.92 ± 0.45</td>
<td>6</td>
<td>49.40 ± 1.43</td>
<td>22</td>
</tr>
</tbody>
</table>

*a* The location is given as the percentage distance from centromere to signal site over the chromosome arm.

*b* The number of chromosomes used for measurements.

three genes representing the bz1 region. The nine single-copy probes were added as a single probe cocktail, which also included CentC and telomere probes, and were hybridized with KYS pachytene chromosomes. While all cells showed hybridization of some of the probes, approximately one-fifth of the observed cells showed the hybridization signals of all probes on chromosome 9 (Figure 4A). Three straightened chromosomes 9 from three meiocytes are lined up in Figure 4B. This probe cocktail produced nine alternate red/green signals of genes and marked centromeres and telomeres on chromosome 9. Even the sh1-tac7077-bz1 region could be resolved as two pairs of red signals derived from sh1 and bz1 probes, and the green signal of tac7077 was present between sh1 and kz1 signals. The yellow fluorescence between the red and green spots represents the region of signal overlap.

Six pachytene chromosomes 9 without morphology distortion showing all hybridization spots were selected for analyzing the locations of signals. We separated the results obtained by the simultaneous FISH mapping from the previous mapping data obtained in the individual FISH experiments and listed the mapping values in Table 3. The locations of each probe obtained from the two sets of FISH experiments were consistent. We combined the data sets to calculate the average positions of the nine probes. This experiment shows that our method is an effective technique for construction of a cytogenetic map.

#### FISH Mapping of Genes on the End of the Genetic Map

We selected three genes that have been genetically mapped to the positions near the termini of linkage maps and determined their physical locations on chromosomes using HR gene FISH. The rough sheath1 (rs1) gene is mapped at 0.0 cM of linkage group 7 on the Genetic 1997 map; however, the FISH results showed that the rs1 gene was located at position 7S.93 (Figures 5A to 5C). absence of first division1 (afd1) is a meiotic mutant and has been mapped to Bin 6.08, which is the last segment of chromosome 6. The mutant phenotype is linked with the simple sequence repeat marker, umc2094, and placed in Bin 2.01, which is the second of the 10 segments of chromosome 2. The kcbp1 fragment amplified from genomic DNA, and pooled with a SS rDNA probe for marking the long arm of chromosome 2, hybridized with KYS pachytene chromosomes. The gene is not located at the terminus of the genetic map; however, the FISH results showed that the kcbp1 gene was located at the terminal region of the short arm of chromosome 2 (Figures 5G to 5I) and gave a precise position at 2S.95. These experiments demonstrate that our HR gene FISH method can be used to investigate the coverage of the genetic map relative to chromosome ends.

#### Detection of Homologies in a Multigene Family Using a Single Probe

The gene zmm1 belongs to the MADS box gene family and has been genetically mapped on chromosome 10S. There have been 32 MADS box genes identified by cDNA screening in maize (Muenster et al., 2002), and the gene zag2 is phylogenetically the most closely related homologue to zmm1 (Theissen et al., 1995). The zag2 gene has been genetically mapped to chromosome 3L and shows 94% identity with zmm1 in the coding region. When we used a 4.1-kb zmm1 genomic DNA fragment that has 72% sequence identity to zag2 genomic DNA for FISH analysis, the brightest signals appeared on chromosome 10S, clearly representing zmm1, and four fainter signals were on other chromosomes with lower detection frequency (Figures 6A and 6B). After reprobing the slide with the marker cocktail to ensure correct identification of all chromosomes, the positions of zmm1 signals were determined to be at 4S.45, 5S.04, 7S.47, 9S.29, and 10S.30 based on the measurement of 13 cells. Because the genomic DNA of zmm1 and zag2 share only 72% homology and the higher similarity is restricted to an ~800-bp coding region, the zag2 on chromosome 3L cannot be detected by zmm1 genomic DNA probe under the high-stringency washing condition.
Identification of Pachytene Chromosomes

The accuracy and efficiency of cytogenetic mapping requires easy-to-use and reliable methods for chromosome identification. The ability to identify the individual pachytene chromosomes from a line of maize depends on the ease of chromosome spreading and on the presence of chromosome landmarks, both of which vary from one genotype to another in maize (Carlson, 1988; Dempsey, 1994). For our work, we used the inbred line KYS, which has long been regarded as superior in its spreading ability and quality of pachytene preparations (Dempsey, 1994). Two groups have reported the identification of mitotic chromosomes of KYS by FISH (Sadder and Weber, 2001; Kato et al., 2004). To establish a reliable FISH mapping system using maize pachytene chromosomes, we developed a marker cocktail that allows unambiguous identification of each pachytene chromosome of KYS and provides confidence in the correct mapping of single-copy genes. The marker cocktail also was hybridized with inbred line B73, which has been used as material for maize genome sequencing, and the hybridization pattern also aided in the identification of the B73 pachytene chromosomes. However, the pachytene chromosomes of B73 are usually tangled with each other, making it difficult to trace individual chromosomes (data not shown). Although not as clearly defined as KYS chromosomes, B73 can be used as material for local cytogenetic mapping in one chromosomal arm to resolve the possible controversies raised from genome projects. Lastly, we were able to strip the signals derived from single-copy probes and then reprobe slides with the marker cocktail, relocate cells previously imaged, and confirm chromosome identity.

Single-Copy Sequence Mapping by HR Gene FISH

The most important result from this work is the ability to locate unique sequences on pachytene chromosomes. This protocol is so robust that we could even simultaneously map nine single-copy sequences, albeit with less efficiency than individual single-copy sequences. The routine detection of single-copy sequences on chromosomes by FISH has been difficult in plants. In maize, Weber and colleagues have localized two single-copy loci, umc105a and csu145a, on chromosome 9 using cosmid clones as probes and Cot-100 DNA as a competitor (Sadder et al., 2000; Sadder and Weber, 2002). Because large genomic clones of maize contain different amounts of repetitive elements, use of Cot DNA to suppress the repetitive sequences in the probe is not successful for every clone, and further work has not ensued. Koumaris and Bass (2003) have used three maize restriction fragment length polymorphism markers to isolate syntenous sorghum BAC clones and have mapped these on maize chromosome 9 in an oat–maize addition line. In their study, the maize chromosome was painted with maize total genomic DNA to distinguish it from the oat chromosomes, and excess unlabeled sorghum total genomic DNA was added in the probe cocktail to block the cross-hybridization. The morphology of maize chromosomes in oat–maize addition lines appears different from native maize pachytene chromosomes, particularly the lack of obvious differentiation between euchromatin and heterochromatin in the pericentromeric region. While the gene order is likely correct, the relative distances between genes may be different in the oat–maize lines.

Several factors contribute to our HR gene FISH protocol. First, the high quality of chromosome preparations derived from a delicate squash protocol is extremely important. The traditional method for cytological chromosome preparations involves heating meiocytes in acetocarmine to a point just before boiling, which makes chromosomes spread but is harsh and leads to a reduction in the strength of the hybridization signals (C.-J.R. Wang and C.-C. Chen, unpublished data). We used a milder

Figure 4. Simultaneous Mapping of Nine Loci as well as Centromere and Telomere Markers on Pachytene Chromosome 9 Using HR Gene FISH.

(A) The HR gene FISH image of nine loci labeled alternately with FITC or Cy3 on pachytene chromosome 9. Signals are indicated by alternate red and green arrowheads. The asterisk indicates the terminal knob on 9S. C, centromere.

(B) Three computationally straightened chromosomes 9 from three independent cells. The left chromosome is straightened from the image shown in (A).Bars = 10 μm.

DISCUSSION

Identification of Pachytene Chromosomes

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squash method, treating anthers with cell wall degrading enzymes followed by acid maceration that yielded better results. Treatment of chromosome preparations with pepsin facilitates probe penetration and reduces background fluorescence (Schwarzacher and Heslop-Harrison, 2000). Second, the use of unique genic regions as probes was paramount. Probes that contained even small amounts of repetitive DNA did not produce observable signals above the cross-hybridization fluorescence. Using unique sequences as probes also eliminated the need to add repetitive blocking DNA. Third, labeling probes directly with fluorescence dyes followed by high-stringency washes and use of a sensitive imaging system boosted the signal-to-noise ratio significantly. We used a deconvolution light microscope system for all slides, but all signals were clearly visible prior to deconvolution. The deconvolution produces a sharper image because it is designed to adjust out-of-focus information back into the correct focal plane and position (Agard et al., 1989), but for this method, a very sensitive CCD camera may be all that is necessary.

When the procedure is performed with these modifications, the detection of unique sequences is then dependent on the size of probes and the homology between probes and targets. We performed the washes at 85% stringency (see Methods), which implies that a hybrid between probes and targets with 85% or more homology will remain stable. Under this condition, the smallest probe we could detect was 3.1 kb. These two criteria, size of probes and the homology between probes and targets, restrict hybridization to almost perfectly matched sequences. For example, the sh1 and sus1 genes sharing 73% similarity in coding regions are deemed to be paralogous genes (Shaw et al., 1994), but there was no cross-hybridization when using their genomic DNA as probes. Another example is the failure to detect zag2 using a zmm1 probe.

Cytogenetic Mapping Resolution on Pachytene Chromosomes by HR Gene FISH

Mapping resolution is mainly determined by the extent of target chromosome condensation. Because maize pachytene chromosomes are 10 to 20 times longer than somatic metaphase chromosomes, pachytene chromosomes provide much better resolution for mapping. For example, the presence of two CentC/A loci in centromere 10 has not been reported in previous studies using FISH on metaphase chromosomes because of the more compact morphology of the target (Sadder and Weber, 2001; Kato et al., 2004). In rice, Cheng et al. (2001b) demonstrated that two BAC clones separated by ~100 kb can be resolved in the euchromatic region of pachytene chromosomes. In tomato, pachytene FISH can resolve two BAC clones separated by 1.2 Mb in heterochromatic regions and 120 kb in euchromatic regions (de Jong et al., 1999). In this study, we found that the tac7077 and bz1 sequences can be resolved in maize KYS pachytene chromosomes and the physical distance between two signals is ~0.26 μm. The limit of resolution of light

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**Figure 5.** Mapping of Genes on the End of the Genetic Map by HR Gene FISH to Demonstrate the Physical Limitation of the Genetic Map.

(A) to (C) The HR gene FISH image of FITC-labeled rs1 fragment (green) and Cy3-labeled centromeres and telomeres (red) on pachytene chromosome 7. (B) shows the magnified image of signals, and the computationally straightened chromosome 7 is shown in (C).

(D) to (F) The HR gene FISH image of FITC-labeled afd1 fragment (green) and Cy3-labeled centromeres and telomeres (red) on pachytene chromosome 6. (E) shows the magnified image of signals, and the computationally straightened chromosome 6 is shown in (F).

(G) to (I) The HR gene FISH image of FITC-labeled kcbp1 fragment (green) and Cy3-labeled 5S rDNA, centromeres, and telomeres (red) on pachytene chromosome 2. (H) shows the magnified image of signals, and the computationally straightened chromosome 2 is shown in (I).

Bars = 10 μm.
microscopy is \( \sim 0.25 \, \mu m \), so the distance between the tac7077 and \( b27 \) loci is probably about the closest that two foci can be resolved. In contrast with the euchromatic location of the tac7077-bz1 region, the loci \( wx1 \) and \( d3 \) are located near the pericentromeric heterochromatin region. The physical distance between \( wx1 \) and \( d3 \) signals on chromosomes is \( \sim 1.74 \, \mu m \) and the molecular distance is 3.6 Mb (F. Wei, personal communication). Based on these observations, the degrees of chromatin condensation in the tac7077-bz1 and \( wx1-d3 \) regions are \( \sim 380 \, kb/\mu m \) and 2 Mb/\( \mu m \), respectively.

### Integrated Cytogenetic Map of Maize Chromosome 9

Our HR gene FISH method for maize provides an opportunity to generate an integrated cytogenetic map. We have correlated the position of genes derived from HR gene FISH and chromosome structure with the UMC98 and IBM genetic linkage maps, the BAC contigs, the breakpoint-based cytogenetic map, and the recombination nodule (RN) map of maize chromosome 9 (Figure 7).

In our comparisons, the order of genes obtained from FISH was concordant with those in the UMC98 and the most recent version of the IBM genetic maps. The cytogenetic map highlights the interesting differences between genetic distance (centimorgans) and cytological distance (micrometers) on a chromosome. According to the UMC98 genetic map, the gene \( sh1 \) has been mapped to 36.4 cM, and the genetic distance between \( sh1 \) and the most distal marker represents 53.5% of the genetic distance on \( 9S \) (Figure 7D). Physically, the \( sh1 \) gene was located in the distal 25% of the length of \( 9S \) (Figure 7C). This implies that half of the crossover events occurs in the last quarter of that chromosome arm. By contrast, in the pericentromeric region, the genetic distance between the markers \( rf2 \) and \( gl15 \) is only 2.9 cm, spanning 1.9% of the total genetic distance of chromosome 9 in the UMC98 map, whereas the physical distance between these two genes represents 28.2% of the total chromosome length. This implies extremely limited crossing over in the pericentromeric region. Significant suppression of genetic recombination in the pericentromeric regions has been reported in maize previously in the cytogenetic maps compiled from translocation data of many researchers (Coe et al., 1993) and in the study of the distribution of RNs (Anderson et al., 2003). A similar situation has also been reported in many organisms, including wheat (Gill et al., 1996), barley (Kunzel et al., 2000), sorghum (Kim et al., 2005), and others (reviewed in Harper and Cande, 2000).

To display the relationship between the genetic and cytological distances along chromosome 9, we calculated the ratio of genetic distance (centimorgans) to cytological distance (micrometers) between genes (Figures 7E and 7F). The ratio varies 145-fold from 7.29 cM/\( \mu m \) near the tip of \( 9S \) to 0.05 cM/\( \mu m \) in the pericentromeric region, demonstrating that the recombination frequency in the distal region of the chromosome is at least 100 times higher than that in the pericentromeric region (Figure 7F). This result is consistent with the RN map of maize (Anderson et al., 2003). RNs are structures that are visible on spreads of pachytene chromosomes by transmission electron microscopy and represent sites of crossovers (Anderson and Stack, 2005). Anderson et al. (2003) measured the absolute position of RNs on hundreds of maize chromosomes 9 and then reported the number found in each 0.2-\( \mu m \) segment, which gives a very good representation of where crossover events occur in general. To compare our centimorgan:micrometer ratios with the average number of RNs along chromosome 9, a red line summarizing the distribution of RNs (Anderson et al., 2003) was superimposed over the bar chart that summarizes our results (Figure 7F). The trends of two charts are similar. The pericentromeric heterochromatin region as defined by DAPI staining also shows a low number of RNs and a low ratio of genetic-to-cytological distance (Figures 7E and 7F). The reduction of recombination in the pericentromeric regions is most likely related to the presence of pericentromeric heterochromatin. The other interesting discovery in this comparison is that recombination frequency at the end of \( 9S \) is approximately twice the rate of that at the end of \( 9L \) in both studies. During meiosis, only one crossover is needed per arm; therefore, the shorter arm has a higher recombination frequency per unit of chromosome length.

The cytological positions of genes also can be assigned to chromosomal segments by genetically mapping them relative to chromosome breakpoints where the position of the breakpoint has been determined by squashes of meiotic prophase or mitotic

**Figure 6.** Detection of Homologs of the MADS Box Gene Family Using the zmm1 Gene as Probe.

(A) Pachytene chromosomes of maize inbred line KYS were hybridized with Cy3-labeled zmm1 genomic fragment (red) and FITC-labeled CentC repeats (green). (B) The computationally straightened chromosomes 4, 5, 7, 9, and 10 taken from the cell shown in (A). Bars = 10 \( \mu m \).
Figure 7. An Integrated Cytogenetic Map of Maize Chromosome 9 Showing Comprehensive Correlation between Gene Positions, Chromosome Structure, Genetic Maps, the BAC Contigs, and the RN Map.

(A) The high-resolution genetic IBM2 map is shown in part (Maize GDB; http://www.maizegdb.org/).

(B) The genetic coverage of 24 BAC contigs anchored on linkage group 9 is shown along the IBM2 map in order (http://www.genome.arizona.edu/fpc/WebAGCol/maize/index.html; new version released on July 19, 2005). The length of the contig corresponds to the position of its corresponding genetic locus on the IBM2 map. Six contigs are anchored to chromosome 9 by nine FISH probes in silico, and the serial numbers of these contigs are listed at left.

(C) Ideogram of pachytene chromosome 9 of maize inbred line KYS based on HR gene FISH mapping results in this study. Heterochromatic and knob regions with bright DAPI staining are shown in dark blue. Green and red circles represent FITC and Cy3 signals, respectively, of FISH generated by the nine-gene probe cocktail. The positions of genes on the chromosome are drawn based on the data in Table 3. The breakpoints of several cytogenetic stocks are indicated by horizontal lines crossing the chromosome. The chromosomal positions of genes and breakpoints are given at right as fractions of chromosome arm length.

(D) UMC98 genetic map redrawn in part from Davis et al. (1999). Highlighted boxes are core markers.

(E) The straightened chromosome 9 from the left image shown in Figure 4B. DAPI staining was converted to black-and-white images and overlaid with signal images. The genetic distances between genes, reported in centimorgans, based on UMC98 map are listed at the left of the chromosome, and the cytological distances between genes, reported in micrometers, are shown at the right of the chromosome.

(F) A comparison of RN distribution along the length of chromosome 9 and the values of centimorgans/micrometers between genes. The chromosome is represented on the y-axis with the short arm on top. The top x-axis is the numbers of RN in 0.2-μm intervals along the chromosome, and the red line shows the general trend of the RN distribution redrawn from Anderson et al. (2003). The horizontal bars represent the ratios of the genetic distance between genes (centimorgans; the value listed at the left of the chromosome in [E]) to the cytological distance between genes (micrometers; the value listed at the right of the chromosome in [E]). The value for each bar is shown at the right of the bar.
metaphase chromosomes (for review, see Harper and Cande, 2000). However, fine mapping of genes on chromosomes using breakpoint analyses is very difficult because markers can only be localized in a chromosome segment. For example, the wx1 gene has been cytogenetically mapped in a chromosomal region between 9S.68 and 9S.43 based on the breakpoint analyses of the T4-9(6222) and T8-9(3630) translocation lines (Figure 7C), whereas the wx1 gene was directly mapped to position 9S.46 by HR gene FISH.

Based on the collected data in MaizeGDB, 19 translocation stocks with breakpoints on 9S have been used for cytogenetic mapping of the wx1 gene. Only one breakpoint analysis, using the TB-9Sb translocation line, is inconsistent with our FISH mapping result, and even here, the position of the breakpoint (9S.40) is close to our placement of the wx1 gene at 9S.46. This inconsistency is likely due to the difficulty in precise identification of the breakpoints. The chromosomal position of the wx1 gene has also been determined by several other approaches. Based on the cumulative RNs observed on chromosomes and the genetic position of wx1, the chromosomal location of the wx1 gene can be predicted to be at position 9S.38 (Morgan2McClintock translator; http://www.lawrencelab.org/Morgan2McClintock/), which is close to our FISH results. Using 3H-labeled probes and autoradiography, Shen et al. (1987) mapped the wx1 gene in maize inbred line 38-11, at a position 32.25% of the chromosome length from the tip of short arm, which can be estimated to position 9S.05 (there was no centromere marker available at that time). The discrepancy of mapping position of the wx1 gene with our data is probably due to the difficulty in accurately determining the locations of silver grains on Giemsa-stained chromosomes and possibly also due to the use of a different inbred line. The wx1 gene was also mapped to 9S.13 in an oat–maize addition line using a sorghum BAC probe (http://www.maizegdb.org/cgi-bin/displaymaprecord.cgi?id=–892372; Koumbaris and Bass, 2003). The inconsistency of this mapping result may be due to small chromosomal rearrangements or the different compactness of maize chromosomes in oat–maize additions, especially in the distinction between heterochromatin and euchromatin.

Applications of HR Gene FISH

HR gene FISH will become a useful tool for maize genome research. We envision a number of uses. First, this method can contribute to the mapping of single-copy genes directly on chromosomes. In addition, our method can help to order BAC contigs and resolve the challenges raised from genome projects. For example, the localization of markers flanking the BAC contigs by FISH will be an effective strategy to estimate the gaps between contigs. The Assembled Zea mays (AZM) sequences are the assembly of methylation filtered and high Cot sequence reads representing 95% of genic sequences in the maize genome, and many of them have been anchored to genetic map and BAC contigs (Springer et al., 2004). Thus, AZM sequences can be used as FISH probes for representing BAC clones or single-copy genes. In this study, two genes, d3 and sbp4, were amplified solely based on AZM4_49750 and AZM4_60110 sequences, which also were found in BAC clones b0177H21 and c0018H12, respectively. Both genes were mapped to precise positions by HR gene FISH.

Second, HR gene FISH will allow the placement of markers that cannot be mapped by recombination-based methods, and rough genetic positions can be assigned by interpolation. Examples of such markers are loci around centromeres, loci that lack polymorphisms in mapping populations, and the multiple gene family members. Although high-density genetic maps, such as the UMC98 map (Davis et al., 1999), containing ~1750 markers have been constructed, many gaps of >10 cM still occur in the terminal and internal regions. Even the IBM2 map, a high-resolution genetic map of maize with a threefold increase in recombination events (Sharopova et al., 2002), still has many gaps >20 cM. HR gene FISH can be used to determine if these gaps are recombination hot spots or large chromosome segments on which markers have not been assigned. Also, this method can be used to estimate the cytological and physical distances between telomeres and the most distal markers on linkage groups to test the physical coverage of genetic maps. In addition, HR gene FISH will provide an accurate approach to assign the centromere position on genetic maps.

In summary, this method will become a powerful tool to construct the cytogenetic map of maize and integrate existing information from different genomic resources to produce a cross-referenced integrated map like we illustrated in Figure 7. It can also be used to address many questions in genome research. The strategy used here for developing HR gene FISH for maize could also be used for constructing cytogenetic maps of other organisms that have large genomes.

METHODS

Plant Material and Fixation

Zea mays inbred lines KYS and B73 were obtained from the National Plant Germplasm System, USDA, and grown in greenhouses at the University of California, Berkeley. For HR gene FISH, meiotic stage tassels were fixed in ethanol-glacial acetic acid (3:1) for 4 to 6 h and stored in 70% ethanol at –20°C; for 3-D FISH, immature anthers were fixed in 4% formaldehyde and stored in buffer A as described by Golubovskaya et al. (2002).

Chromosome Preparations

In the HR gene FISH method, meiotic chromosome spreads were obtained as described by Chen et al. (2000) with some modifications. Briefly, anthers containing pachytene-stage meiocytes were treated with 2% (w/v) Onozuka R10 cellulase (Yakult Honsha), 1% (w/v) Macerozyme R10 (Yakult Honsha), and 1% (w/v) cytolyticase (Sigma-Aldrich) in 4 mM citric acid/6 mM sodium citrate buffer, pH 4.8, at 25°C for 20 to 40 min. Meiocytes were squeezed into a drop of 45% acetic acid. Anther wall debris was removed and then a cover slip was added. The slide was placed upside down on filter paper, and the chromosomes were squashed by applying gentle pressure to the slide. The cover slip was removed with a razor blade after freezing the slide in liquid nitrogen. Slides were then dehydrated in 50, 70, and 100% ethanol.

For 3-D FISH, the meiocytes were embedded in polyacrylamide pads as described by Golubovskaya et al. (2002). Briefly, meiocytes released from fixed anthers were suspended in buffer A, and the acrylamide pad was prepared on a glass cover slip by addition of 5 μL activated acrylamide stock (5 μL of 20% ammonium persulfate, 5 μL of 20% sodium sulfide, 50 μL of a 30% 29:1 acrylamide: bisacrylamide solution, and 50 μL of
2X buffer A) into 10 μL meiotic suspension, and a second cover slip was placed on top for 45 min. The cover slips were taken apart with a razor blade.

**Marker Cocktail Constitution**

The following six repeated DNA sequences were used for karyotyping: Cy3-labeled Cent C (Ananiev et al., 1998b), Cy5-labeled telomere sequences, FITC-labeled 180-bp repeats (Peacock et al., 1981), Cy3- and Cy5-labeled TR-1 elements (Ananiev et al., 1998a; Hsu et al., 2003), FITC- and Cy5-labeled SS ribosomal DNA, and FITC- and Cy3-labeled 4SS rDNA. The CentC and SS rDNA clones were obtained by PCR amplification. The telomere sequences were amplified by PCR in the absence of template using oligonucleotides 5'-(TTTAGGG)4 and 5'-(CCCTAAA)4 as primers (Cox et al., 1993). These sequences were all labeled by nick translation.

**Cloning of Gene Probes**

Twenty-one DNA fragments were used as gene probes for FISH (see Supplemental Table 2 online). Seven clones were generously provided by the maize community as listed in Supplemental Table 2 online. Fourteen fragments were amplified from KYS genomic DNA based on sequence information and the primers are listed in Supplemental Table 2 online. Seven clones were generously provided by Dooner for the pHF10 clone, Curtis Hannah for the sh1 probe, Victor Llaca for the zein probe, Randall Tyers for the afd1 clones, Olivier Hamant for the 180-bp knob probes, and Yen-Yu Kao for the 4SS rDNA clone. These data sets were subjected to deconvolution. For determining the physical position of signals, only chromosomes without apparent morphological distortion were analyzed. The path of the chromosome was computationally traced and straightened, and then the physical position of signals was measured. Final image adjustments were done with Adobe Photoshop 5.0 software.

**FISH**

For HR gene FISH, slides with good chromosome spreads were pre-treated with 100 μg/mL DNase-free RNAsin (Sigma-Aldrich) in 2X SSC (0.3 M NaCl and 30 mM trisodium citrate) at 37°C for 30 min and then washed three times for 5 min in 2X SSC. For removing the cyttoplasm, the slides were treated with 1 μg/mL pepsin (Sigma-Aldrich) in 10 mM HCl for 20 to 60 min at 37°C and then placed in distilled water for 1 min to stop the reaction and washed three times in 2X SSC for 5 min. Subsequently, slides were treated in 4% formaldehyde for 10 min at room temperature, washed three times in 2X SSC for 5 min, and dehydrated in 70, 90, and 100% ethanol.

The chromosomal slides were denatured in 70% formamide in 2X SSC at 72°C for 2.5 min and dehydrated in prechilled 70, 90, and 100% ethanol. The hybridization mixture consisted of 50% formamide, 2X SSC, 10% dextran sulfate, 0.2% SDS, 5 ng/μL DNA for each probe, and 0.5 μg/μL herring sperm DNA and was denatured at 85°C for 10 min. Hybridization was performed at 37°C for 24 to 30 h, followed by washes in 2X SSC, 0.2X SSC, 20% formamide in 0.1X SSC, 0.2X SSC, and 2X SSC at 42°C for 10 min each. According to the formula of Meinkoth and Wahl (1984), melting temperature (Tm) = 81.5 + 0.41 (%GC content of probe) + 16.6 log (molarity of monovalent ions) − 500/(probe fragment length) − 0.61 (%formamide), the Tm of probe under hybridization and wash conditions can be calculated. The stringency of our FISH experiments can be further determined by agarose gel electrophoresis, cloned by TOPO TA cloning kits (Invitrogen), and confirmed by partial sequencing and restriction enzyme mapping.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Mapping of Two Centromeric Sequences, CentA and CentC, on Pachytene Chromosomes of KYS.

**Supplemental Figure 2.** Mapping of 22-kd α-Zein Cluster on Pachytene Chromosome 4 Using HR Gene and 3-D FISH.

**Supplemental Figure 3.** The Effect of Pepsin Treatment on Maize KYS Pachytene Chromosomes.

**Supplemental Table 1.** The Difference of Length between Mitotic Metaphase and Meiotic Pachytene Chromosomes.

**Supplemental Table 2.** Gene Probes Used in This Study.

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