Centromeric DNA sequences in multicellular eukaryotes are often highly repetitive and are not unique to a specific centromere or to centromeres at all. Thus, it is a major challenge to study the fine structure of individual plant centromeres. We used a DNA fiber-fluorescence in situ hybridization approach to study individual maize (Zea mays) centromeres using oat (Avena sativa)-maize chromosome addition lines. The maize centromere-specific satellite repeat CentC in the addition lines allowed us to delineate the size and organization of centromeric DNA of individual maize chromosomes. We demonstrate that the cores of maize centromeres contain mainly CentC arrays and clusters of a centromere-specific retrotransposon, CRM. CentC and CRM sequences are highly intermingled. The amount of CentC/CRM sequence varies from \( \sim 300 \) to \( >2800 \) kb among different centromeres. The association of CentC and CRM with centromeric histone H3 (CENH3) was visualized by a sequential detection procedure on stretched centromeres. The analysis revealed that CENH3 is always associated with CentC and CRM but that not all CentC or CRM sequences are associated with CENH3. We further demonstrate that in the chromosomal addition lines in which two \( \text{CenH3} \) genes were present, one from oat and one from maize, the oat CENH3 was consistently incorporated by the maize centromeres.

INTRODUCTION

The centromere is the chromosomal domain responsible for kinetochore formation and sister chromatid cohesion. Thus, centromeres play the key role in faithful transmission of chromosomes during mitosis and meiosis. In multicellular eukaryotes, centromeres are embedded within cytologically distinctive heterochromatin and are associated with long tracts of repetitive DNA. Satellite DNA and retrotransposons are the most common DNA elements found in plant centromeres (Jiang et al., 2003). The centromere-specific satellite repeats reported in several plant species show a structure and organization that is similar to the \( \alpha \) satellite in human centromeres (Round et al., 1997; Gindullis et al., 2001; Cheng et al., 2002b). Data from several species suggest that satellite DNA has a functional role in chromosome segregation (Kaszas and Birchler, 1996, 1998; Cheng et al., 2002b; Zhong et al., 2002; Nagaki et al., 2003b, 2004). Among the retroelements known at plant centromeres, the centromeric retrotransposon (CR) family in Gramineae species is most intriguing. Unlike other retrotransposon families that diverge rapidly during evolution, the CR family has been found in the centromeres of all Gramineae species studied (Aragon-Alcaide et al., 1996; Jiang et al., 1996; Miller et al., 1998; Presting et al., 1998; Langdon et al., 2000). Highly conserved motifs were found in the long terminal repeats of the CR elements from rice (Oryza sativa), maize (Zea mays), and Hordeum vulgare (barley) (Nagaki et al., 2003a). Recent data from maize showed that the CR elements may play a critical role in centromere function (Zhong et al., 2002).

Whereas there are no universally conserved DNA sequences associated with centromere function, several proteins specific to the centromere/kinetochore complex are highly conserved (Dobie et al., 1999; Yu et al., 2000; Houben and Schubert, 2003). A centromere-specific histone H3-like protein, referred to as CENH3, has been found to underlie the kinetochore (see reviews in Henikoff et al., 2001; Sullivan et al., 2001). The first CENH3, CENP-A (centromeric protein A), was identified in humans (Palmer et al., 1987, 1991). Since then, CENH3 has been found in all model eukaryotes (Henikoff et al., 2001), including Arabidopsis (Arabidopsis thaliana) (Talbert et al., 2002), maize (Zhong et al., 2002), and rice (Nagaki et al., 2004). CENH3s replace the regular histone H3 in centromeric chromatin (Yoda et al., 2000). There are numerous lines of evidence that CENH3 plays the key role in the establishment and function of kinetochores in various organisms. CENP-A is present only in
the functional centromeres of cytologically stable dicentric chromosomes in humans (Warburton et al., 1997). CENH3 also is present in significantly rearranged centromeres and human neocentromeres (Sullivan et al., 2001). Inactivation of CENH3 proteins in yeasts, worms, flies, and mammals severely disrupts mitosis and cell-cycle progression and causes mislocation of many other centromere/kinetochore proteins (Sullivan et al., 2001). All of these data indicate that CENH3 is a central component in kinetochore formation and centromere function.

Maize has become an important model for plant centromere research. Maize centromeres contain a 156-bp satellite repeat, CentC, and the centromere-specific retrotransposon CRM (the maize subfamily of CR) (Ananiev et al., 1998; Chen et al., 2000; Zhong et al., 2002; Nagaki et al., 2003a). Both CentC and CRM sequences interact with CENH3, indicating that they are located within the functional maize centromeres (Zhong et al., 2002). Two BAC clones derived from maize centromeres have recently been sequenced (Nagaki et al., 2003a). These two BACs contain primarily CentC and retrotransposon sequences. Alfenito and Birchler (1993) also isolated a repeat specific to the centromeres of maize B chromosomes. The B-specific repeat is thought to reside in the functional portion of the B centromere based on the fact that centromere misdivision derivatives, resulting from a break on the short arm and subsequently on the long arm side of the centromere, still retain substantial B repeat copies. The B centromere repeat is homologous to yet another maize repeat, Cent4, which is only found on chromosome 4 (Page et al., 2001).

Despite the extensive work on maize centromeres, the large-scale DNA structure and organization of individual maize centromeres is not known. In this study, we used the recently developed oat (Avena sativa)-maize addition lines (Kynast et al., 2001) to study individual maize centromeres. We demonstrate that the cores of seven maize centromeres consist almost exclusively of intermingled CentC and CRM sequences. The quantities of CentC and CRM and their organization relative to each other vary significantly among different maize centromeres. The structural relationship between CENH3 and CentC/CRM was visualized by sequential detection of the protein and centromeric DNA on stretched centromeres. We also demonstrate that maize centromeres consistently adopt the oat CENH3 after being transferred into the oat genetic background.

RESULTS

CentC and CRM Are Located in the Cytologically Defined Maize Centromeres

We conducted fluorescence in situ hybridization (FISH) mapping of CentC and CRM sequences on maize chromosomes at different meiotic stages. On the highly extended pachytene chromosomes, both CentC and CRM sequences were restricted to the primary constriction regions. The FISH signals were located at the most poleward positions on chromosomes at metaphase I and anaphase I stages (Figures 1A and 1B). These results confirm that CentC and CRM are located at the cytologically defined maize centromeres (Ananiev et al., 1998; Zhong et al., 2002).

DNA Organization of Individual Maize Centromeres

Oat-maize chromosome addition lines (Kynast et al., 2001) were used to study the DNA organization of individual maize centromeres. FISH analysis using CentC and CRM probes confirmed the presence of a single (monosomic addition) or a pair (disomic addition) of maize chromosomes in the seven oat-maize addition lines used in this study (Figures 2A to 2D). Cross-hybridization of the CRM probe to oat centromeres was observed (Figure 2C). However, the CentC probe was highly specific to the maize chromosome (Figure 2B), which facilitates unambiguous identification of fiber-FISH signals derived from the CentC and CRM probes.

The distributions of CentC and CRM sequences in the centromeres of maize chromosomes 1, 2, 3, 4, 6, 7, and 9 were analyzed using fiber-FISH. A minimum of eight digital images of the fiber-FISH signals were collected from each centromere and measured using IPLab software. The lengths of the fiber-FISH signals (in micrometers) were converted to kilobases using a 3.21-kb/μm conversion rate (Cheng et al., 2002a) (Table 1). In general, the fiber-FISH signals derived from the CentC and CRM probes were contiguous with few unambiguous gaps, suggesting that these two sequences are the dominant DNA components of maize centromeres (Figures 2E to 2I and 3). Interspersion of the two sequences was observed in all seven maize centromeres (Figures 2E to 2I and 3).

We divided the fiber-FISH signals into a central domain and two flanking domains. The two ends of each central domain are marked by distinct CentC signals (Figures 2F, 2G, and 2I). These end CentC signals are usually flanked by additional CRM signals. The sizes of the fiber-FISH measurements, which include both central and flanking domains, varied from 299 kb ± 29 kb of centromere 3 to >2.8 Mb of centromere 7 (Table 1). Technically, it is difficult to obtain DNA fibers >1 Mb. Thus, most of the fiber-FISH signals of centromeres 1 and 7 may be derived from broken DNA fibers. We used the longest fiber-FISH signals to represent the minimum size of these two centromeres (Table 1).

The relative amount of CentC and CRM sequences within each centromere was calculated by counting the numbers of green

![Figure 1. Cytological Localization of CentC and CRM Sequences on Meiotic Chromosomes of Maize Inbred B73.](image)

(A) Metaphase I. Both CentC and CRM are located on the most poleward position on each bivalent chromosome.

(B) Anaphase I.

CentC, green; CRM, red. Bars = 10 μm.
Figure 2. Distribution of CentC and CRM Sequences in Individual Maize Centromeres.

(A) to (D) FISH mapping of CentC (green) and CRM (red) sequences on somatic metaphase chromosomes of oat-maize addition line 6.
(A) 4′,6-diamidino-2-phenylindole staining of a somatic metaphase cell.
(B) FISH signals derived from CentC (arrow).
(C) FISH signals derived from CRM.
(D) A merged image of the chromosomes and the FISH signals. Arrow points to the CentC signal.
(E) to (I) Fiber-FISH signals of CentC (green) and CRM (red) from the centromeres of maize chromosomes 1, 2, 3, 7, and 9, respectively. Arrows point to the borders of the central domains of the fiber-FISH signals.
Bars = 10 μm in (D), (F), (G), and (I), 20 μm in (E), and 50 μm in (H).
and red fluorescence spots in each digital image. The CRM sequences account for 26 to 60% of the fiber-FISH signals in the seven centromeres (Table 1). The CRM signals were as short as one to three consecutive fluorescence spots, which may represent a single CRM element, to loose clusters as long as 350 μm (>1000 kb) (data not shown). Although the signals derived from CentC and CRM in the central domains were largely contiguous, unambiguous gaps can be identified in centromeres 2, 4, and 9. These gaps likely represent DNA sequences other than CentC and CRM.

To investigate whether the organization of the CentC and CRM sequences were rearranged during and/or after the maize chromosomes were transferred into the oat background, we conducted fiber-FISH analysis on three independently isolated oat-maize chromosome 6 addition lines (lines 6.1, 6.5, and 6.18). The fiber-FISH signal patterns derived from the three centromeres were nearly identical (Figure 3). These data suggest that the transfer of a maize chromosome into oat does not cause substantial changes in the content or organization of the centromere.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>n</th>
<th>Left Flanking CRM (kb)a</th>
<th>Central Domain (kb)b</th>
<th>Right Flanking CRM (kb)a</th>
<th>Full Size (μm)</th>
<th>Full Size (kb)c</th>
<th>CentC (%)</th>
<th>CRM (%)d</th>
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<tr>
<td>1</td>
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<td>ND</td>
<td>≥1720</td>
<td>ND</td>
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<td>≥1720</td>
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<td>29.6 ± 3.9</td>
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<td>2</td>
<td>18</td>
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<td>282 ± 15</td>
<td>≥69 ± 29</td>
<td>≥121 ± 11</td>
<td>≥388 ± 34</td>
<td>70.6 ± 3.7</td>
<td>29.4 ± 3.7</td>
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<td>241 ± 24</td>
<td>≥6 ± 3</td>
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<td>≥164 ± 45</td>
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<td>≥52 ± 25</td>
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<td>≥160 ± 29</td>
<td>≥221 ± 15</td>
<td>≥709 ± 47</td>
<td>48.4 ± 4.7</td>
<td>51.6 ± 4.7</td>
</tr>
</tbody>
</table>

a The end of the flanking CRM domains cannot be determined unambiguously. We used the most characteristic CRM signals at each end for the measurements that represent the minimum sizes of these flanking domains. ND, no data.
b The size of the core domain of centromeres 1 and 7 cannot be determined. The longest fiber-FISH signals were used as the minimum sizes of these domains.
c The lengths of the fiber-FISH signals (μm) were converted to kilobases using a 3.21-kb/μm conversion rate (Cheng et al., 2002a).
d The percentage of the CentC and CRM sequences are relative to each other without including the unknown sequences in the centromeres.

Figure 3. Distribution Patterns of CentC and CRM Sequences in the Centromeres of Maize Chromosome 6 from Three Independently Isolated Oat-Maize Chromosome 6 Addition Lines.
CentC, green; CRM, red. Bars = 10 μm.
Centromere 4 Is a CRM-Rich Domain That Does Not Include the Cent4 Repeat

In the original description of CentC, the authors noted that chromosome 4 has an unusually small amount of CentC (Ananiev et al., 1998). Page et al. (2001) later identified a repeat localized to the primary constriction of chromosome 4, called Cent4. Here, we tested the hypothesis that Cent4 may substitute for CentC and extend the size of the functional centromere.

We found that the fiber-FISH pattern of CentC and CRM on chromosome 4 is similar to those from other maize centromeres. The intermingled CentC/CRM sequences span ~532 kb ± 74 kb and consist of ~60% CRM and ~40% CentC (Figure 4A, Table 1). The total amount of CentC in centromere 4 was estimated at ~100 kb, less than any of the other six chromosomes analyzed.

Fiber-FISH signals derived from Cent4 were divided into three arrays with different sizes (Figure 4B). The Cent4 locus spans 311 kb ± 25 kb DNA (including the two gaps) based on 14 fiber-FISH measurements. We were not able to find an unambiguous connection between signals derived from Cent4 and CentC on extended DNA fibers, suggesting that these two sequences are separated by >500 kb, the detection limit of fiber-FISH (Jackson et al., 1998). Supporting this view is the fact that the Cent4 and CentC signals were clearly separated from each other on pachytene chromosomes (Figure 4C) and that the signals from Cent4 always lagged behind CentC on centromeres drawn poleward on the spindle (Figure 4D).

We also conducted chromatin immunoprecipitation (ChIP) analysis to test whether the Cent4 sequence interacts with maize CENH3. Data from three independent experiments showed that whereas CentC and CRM were communoprecipitated at high levels, Cent4 was not (Figure 5). Taken together, the available data indicate that the major cluster of Cent4 does not associate with CENH3.

Nearly All CENH3 Is Associated with CentC and CRM, but Not All CentC and CRM Sequences Are Associated with CENH3

Data from human neocentromeres showed that CENP-A–associated chromatin domains contain ~130 to 460 kb DNA (Lo et al., 2001a, 2001b; Alonso et al., 2003). This is roughly consistent with the lengths of the CentC/CRM domains on chromosomes 2, 3, 4, 6, and 9 but substantially less than the length of apparent centromeric DNA on chromosomes 1 and 7.
Only a portion of the unusually long centromeric domains of chromosomes 1 and 7 may interact with CENH3. To test this hypothesis, we developed a technique to sequentially visualize CENH3 and CentC/CRM sequences on stretched chromatin fibers. Interphase nuclei isolated from freshly grown maize callus were used for chromatin fiber preparation (see Methods). Chromatin from purified nuclei was gently stretched, fixed, and treated with the maize antibody to CENH3. Immunostaining signals derived from well-stretched centromeres were recorded. The same slides then were probed with CentC and CRM for fiber-FISH analysis. We frequently observed fiber-FISH signals that spanned regions not covered by anti-CENH3 signals (Figure 6).

However, we rarely observed the opposite of this: chromatin fibers that were stained by CENH3 antibodies nearly always coincided with CentC/CRM sequences. Three types of colocalization of CENH3 and CentC/CRM were observed among 25 centromeres analyzed: (1) a nearly perfect overlap of CENH3 and CentC/CRM sequences (13 cases); (2) CENH3 staining located in the middle of the fiber-FISH signals (2 cases); and (3) CENH3 staining located to one end of the fiber-FISH signals (10 cases; Figure 6).

Functional Adaptation of Maize Centromeres in the Genetic Background of Oat

We previously identified a full-length cDNA from the maize CenH3 gene (Zhong et al., 2002). Physical mapping data indicate that this single maize CenH3 gene (marker CL12047-1) lies close to umc1762 on chromosome 6 (http://genome.arizona.edu/fpc/maize/). To verify this map location, we probed DNA gel blots from the 10 oat-maize addition lines with the CenH3 cDNA. As expected, strong hybridization was only observed in the chromosome 6 addition line (data not shown). Thus, this strain contains both the oat and maize CenH3 genes. We wondered whether maize CENH3 might preferentially associate with the maize centromere, as might be expected if CENH3 has sequence-specific binding properties (Malik and Henikoff, 2002).

Three independently isolated oat-maize chromosome 6 addition lines (lines 6.1, 6.5, and 6.18) were immunoassayed using the maize-specific antibody to CENH3. Root tip cells from maize and oat-maize chromosome 6 addition lines were mixed in the preparations. After the preparations were stained with the CENH3 antibody, the same slides then were used for genomic in situ hybridization using maize genomic DNA as a probe to confirm the presence of the maize chromosomes. No CENH3 staining was apparent in all three chromosome 6 addition lines (Figures 7A and 7B). The immunostaining data raised the possibility that maize CenH3 is not expressed in the oat background. Reverse transcriptase (RT) PCR was used to test this hypothesis. As shown in Figure 8, CenH3 transcripts were readily detected in the maize Seneca 60 inbred line, but they...
Figure 7. Incorporation of Oat CENH3 in Maize Centromeres.

(A) and (B) Maize CENH3 is not detected in the genetic background of oat. A cytological preparation containing root tip cells from both maize and oat-maize addition line 6 was immunostained by the maize anti-CENH3 antibody (A). Immunostaining signals only were observed on the two maize nuclei. The same cells were hybridized to a maize genomic DNA probe, confirming the presence of the two maize chromosomes 6 in the two nuclei derived from oat-maize addition line 6 (B).

(C) Immunostaining of maize chromosomes by the anti-CENH3 antibody developed in rice.

(D) Immunostaining of oat chromosomes by the anti-CENH3 antibody developed in rice.

(E) and (F) Incorporation of oat CENH3 by the centromere of maize chromosome 6. Immunostaining of chromosomes from an oat-maize chromosome addition 6 by the anti-CENH3 antibody developed in rice (E). Signals were visible on all oat chromosomes and the two maize chromosomes (arrows). The same cell was hybridized to a maize genomic DNA probe to facilitate the identification of the two maize chromosomes (F).

Bars = 10 μm.
could not be detected in the oat-maize chromosome 6 addition line that carries the same CenH3 gene from Seneca 60. This result suggests that maize CenH3 is silenced in oat.

We also produced antibodies to rice CENH3 (Nagaki et al., 2004). The rice antibodies stain the centromeres of maize, oat, and several other Gramineae species (Figures 7C and 7D). Maize chromosome 6 and maize chromosomes in the other six addition lines showed clear immunostaining signals derived from the rice antibody (Figures 7E and 7F). These results confirm that the maize centromeres have incorporated the oat CENH3 in the addition lines.

**DISCUSSION**

**Organization, Abundance, and Incorporation of CentC/CRM Sequences in CENH3-Associated Chromatin in Maize Chromosomes**

Maize centromeres contain CentC satellite arrays and CRM elements that interact with maize CENH3 (Zhong et al., 2002). Other retroelements are rare within maize centromeres (Mroczek and Dawe, 2003) and do not interact with CENH3 (Zhong et al., 2002). Likewise, CENH3 does not associate with Cent4, which is present at or near the primary constriction of chromosome 4 (Page et al., 2001) (Figures 4 and 5). Here, we confirm the previous interpretations by showing that nearly all CENH3 is associated with CentC and CRM. We also show that fiber-FISH signals derived from CentC and CRM probes are largely contiguous (Figures 2E to 2I and 3), and the CentC and CRM sequences are highly intermingled throughout all seven maize centromeres analyzed (Figures 2 and 3). Notably, some maize centromeres contain more CRM sequences than the CentC satellite in the core domain (Table 1).

It is interesting to note that the centromeres of maize chromosomes 2, 3, 4, 6, and 9 contain similar amounts of the CentC/CRM sequences, ranging from ~300 to ~700 kb (Table 1). This size range is consistent with the sizes of reported functional centromeres in a variety of other species. The centromere of a Drosophila melanogaster minichromosome is contained within a 420-kb region of centric repetitive DNA (Murphy and Karpen, 1995; Sun et al., 1997). Similarly, the CENP-A–associated regions in a sampling of human neocentromeres span ~130 to 460 kb DNA (Lo et al., 2001a, 2001b; Alonso et al., 2003), and a centromere misdivision study in maize suggested that 500 kb of centromeric repeats is the minimum for fully functional B centromeres (Kaszas and Birchler, 1998). The CentC/CRM cores of maize centromeres 2, 3, 4, 6, and 9 may be large enough for formation of full-sized kinetochores.

Sequential detection of CENH3 and the CentC/CRM sequences on stretched chromatin fibers confirmed the interaction that was previously revealed by ChIP analysis (Zhong et al., 2002). We rarely observed chromatin fibers that were stained by CENH3 antibodies but were not associated with CentC/CRM sequences, suggesting that the intermingled CentC/CRM sequence domains account for the majority of the CENH3-associated centromeric chromatin in maize. We also demonstrate that not all of the CentC/CRM sequences are associated with CENH3 (Figure 6), supporting the view that the recognition of centromeric DNA by CENH3 is an epigenetic event. We noticed that the CENH3-associated domains are often located near one end of the CentC/CRM sequences. Similarly, the functional domain of the centromere of human X chromosome is located near the short arm edge of the α satellite array DXZ1 (Spence et al., 2002).

**Oat CENH3 Functions as a Kinetochore Protein on Maize Centromeres**

CENH3s share high homology with the regular histone H3 in the core domain, but they diverge significantly in the N-terminal tail and the Loop 1 region of the histone core (Henikoff et al., 2001). The diverged regions in CENH3s interact with DNA in the nucleosome and show evidence of adaptive evolution (Malik and Henikoff, 2001; Talbert et al., 2002). The anti-CENH3 antibody of A. thaliana does not stain the centromeres in the closely related species A. arenosa but recognizes all centromeres in both synthetic and natural allopolyploids derived from these two species. These results showed that the CENH3 encoded by A. thaliana was successfully incorporated into the centromeric nucleosomes of both A. thaliana and A. arenosa genomes in the allopolyploids, although the centromeric satellite repeats of these two species share only 58 to 80% sequence identity (Kamm et al., 1995). Talbert et al. (2002) suggested that this may be a consequence of the formation of nucleosomes that include CENH3 monomers from both parental genomes and therefore are able to interact with both sets of centromeric repeats. This idea has yet to be tested because the authors did not have the antisera to A. arenosa CENH3 that would have allowed them to determine whether both forms of CENH3 are present at a single centromere.

In this study, we used antibodies developed to both maize CENH3 and to rice CENH3. These two antibodies were...
developed against peptides located in the different regions of the N-tails (Zhong et al., 2002; Nagaki et al., 2004). The maize CENH3 antibody is specific for maize, but the rice antibody stains the centromeres of rice as well as several other cereal species, including oat and maize (Figure 7). We demonstrated that the CenH3 gene in maize is located on chromosome 6 and that maize CENH3 was not detected in three independently isolated oat-maize chromosome 6 addition lines. However, the maize centromeres in different oat-maize addition lines were recognized by the antibody to rice CENH3. Thus, the centromeric chromatin in both oat and maize chromosomes incorporated only the CENH3 encoded by the oat CenH3 gene. These data and the fact that the maize chromosomes are stable in the genetic background of oat clearly indicate that oat CENH3 is itself sufficient to organize a kinetochore on a maize chromosome.

Rapid elimination of chromosomes from one parental genome is a common phenomenon in somatic and sexual hybrids derived from distantly related species. Elimination of maize chromosomes occurred in sexual hybrids between maize and several cereal species, including *Triticum aestivum* (wheat) (Laurie and Bennett, 1986; O’Donoughue and Bennett, 1994), *H. vulgare* (Laurie and Bennett, 1988; Chen et al., 1991), and oat (Rines and Dahleen, 1990). The chromosome elimination occurred in the first three cell-division cycles in the zygotes of *T. aestivum* × maize hybrids (Laurie and Bennett, 1989). It was noted that the maize chromosomes had small or indistinguishable centromeres and tended to lie away from the plane of the metaphase plate. Laurie and Bennett (1989) suggested that the elimination of maize chromosomes may be caused by impaired centromere function. Laurie and Bennett (1988) also noticed that the maize chromosomes in crosses between *H. vulgare* and maize had well-differentiated centromeres in zygotes and were retained for more cell cycles than in *T. aestivum*.

Riera-Lizarazu et al. (1996) reported that 30 out of 90 plants derived from oat × maize crosses retained one to four maize chromosomes, resulting from incomplete maize chromosome elimination in the hybrids. Plants with chromosomal chimeras, in which some tissues of the plants contain maize chromosomes and other tissues do not, also were recovered, suggesting that maize chromosome elimination in oat × maize crosses is more gradual in comparison to *T. aestivum* × maize crosses (Riera-Lizarazu et al., 1996). By contrast, retention of maize chromosomes was not reported in numerous attempts in the *T. aestivum* × maize crosses conducted by several different laboratories. It is not known if the centromeric repeats of oat and maize share more sequence similarity than, for instance, the centromeric repeats of *T. aestivum* and maize. Alternatively, oat and maize CENH3s may share critical amino acid identities in regions that contact DNA that are absent in *T. aestivum* CENH3. In either case, the data would support the view that CENH3 has evolved to recognize specific centromere repeats (Malik and Henikoff, 2002). It would be interesting to conduct immunostaining experiments on chromosomes in the zygotes derived from the *T. aestivum* × maize and oat × maize crosses. Such experiments may reveal if the failure/success of incorporation of the *T. aestivum* × oat CENH3s in maize centromeres play a role in the elimination of maize chromosomes in the hybrids.

**METHODS**

**Materials**

Oat-maize chromosome addition lines 1, 2, 3, 4, 6 (including lines 6.1, 6.5, and 6.18), 7, and 9 were provided by Howard Rines and Ronald Phillips of the University of Minnesota. Maize inbred lines B73, W22, and Seneca 60 and oat cultivar Starter-1 were used for molecular and cytological analyses. The centromeric DNA probes, including CentC, CRM, and Cent4, all were described previously (Page et al., 2001; Nagaki et al., 2003a).

**FISH and Fiber-FISH**

The FISH and fiber-FISH procedures were essentially the same as the published protocols (Jiang et al., 1995; Jackson et al., 1998). DNA probes were labeled with either biotin-dUTP or digoxigenin-dUTP (Roche, Indianapolis, IN). Chromosomes were counterstained by 4’,6-diamidino-2-phenylindole in the antifade solution Vectashield (Vector Laboratories, Burlingame, CA). All of the images were captured digitally using a SenSys CCD camera (Roher Scientific, Tucson, AZ) attached to an Olympus BX60 epifluorescence microscope (Tokyo, Japan). The camera control and measurements of fiber-FISH signals were performed using IPLab Spectrum software version 3.1 (Signal Analytics, Vienna, VA).

**ChiP**

ChiP was performed as previously (Zhong et al., 2002) except that nuclei from young kernels of the inbred W23 were treated with RNase-free DNase (Promega, Madison, WI) for 10 min at 37°C, using a concentration of 4 units/mg of DNA. Fragments in the size range of 300 to 800 bp were used for immunoprecipitation.

**Immunostaining on Somatic Metaphase Chromosomes**

Root tips were harvested from actively growing plants and fixed immediately using 4% paraformaldehyde in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl2, and 0.3 mM sorbitol, pH 6.8) for 20 min. After washing with PBS (10 mM sodium phosphate, pH 7.0, and 140 mM NaCl), the root tips were treated with 2% cellulase and 1% pectinase (Sigma, St. Louis, MO) in PHEM for ~2 h and then squashed on slides coated with 3-aminopropyltriethoxysilane. The rabbit antibodies to CENH3 (Biosource International, Camarillo, CA) were diluted to 1:2000 in TNT buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent) (Upstate Biochemicals, Lake Placid, NY). Approximately 100 μL of the diluted antibodies was added to each slide, and the slides were incubated in a humid chamber at 37°C for 3 h. After three washes in PBS, 100 μL of rhodamine-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) (1:50 in TNT buffer) was added to the slides. Incubation and washes were the same as for the primary antibody. The slides were counterstained with 4’,6-diamidino-2-phenylindole before being checked with a microscope.

**Sequential Detection of CENH3 and CentC/CRM Sequences on Stretched Centromeres**

Preparation of stretched chromatin fibers was performed according to Blower et al. (2002) with some modifications. Actively growing maize callus (2 weeks old) was ground with liquid N2, and the powder was resuspended in Tris–buffered saline containing 0.5% Tween 40. The nuclei were purified in a sucrose gradient. Extended chromatin fibers...
were prepared by centrifuging the nuclei (in 75 mM KCl) onto slides at 1000 rpm for 5 min using Cytospin 4 centrifuge (Shandon, Pittsburgh, PA). The nuclei on the slide were treated with 100 μL of lysis buffer (25 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100, and 0.2 M urea) covered with parafilm for 10 min. The parafilm was slowly dragged down the slide. The slides then were fixed in cold methanol for 10 min and were incubated in TNB buffer for 30 min at room temperature before immunostaining. The immunostaining procedure on stretched chromatin fibers was the same as that used on somatic metaphase chromosomes. After recording the immunostaining signals, the slides were washed in PBS buffer three times (5 min each) and dehydrated in an ethanol series. These slides were probed with CenTc and CRM for FISH analysis.

RT-PCR

DNA and total RNA was extracted from leaf tissue of 10-d-old plants using the DNeasy and RNeasy plant mini kits (Qiagen, Valencia, CA). RNA samples were treated with RNase-free DNase (Ambion, Austin, TX). cDNA was synthesized using the RNA and Superscript III RT (Invitrogen, Carlsbad, CA). The primer pairs used for PCR and RT-PCR were CenH3-1 (5’-GGGAGGATCGAGGATCGGATC-3’) and CenH3-2 (5’-GATTCG-CCATTCCAAAAGCTTG-3’), which were designed from two different exons within the maize CenH3 gene.

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