B Chromosome Behavior in Maize Pollen as Determined by a Molecular Probe

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ABSTRACT

The B chromosomes of maize typically undergo nondisjunction during the second microspore division (generative cell division). When the microspore nucleus contains only one B chromosome, two kinds of sperm result, one with two B chromosomes and one with no B chromosomes. The sperm with the B chromosomes preferentially fertilizes the egg cell. Previous studies of these phenomena have been limited to genetic analysis and chromosome spreads. In this study we show that a B chromosome-specific probe can be used with fluorescence in situ hybridization (FISH) analysis to detect the presence, location, and frequency of B chromosomes in intact interphase nuclei within mature pollen of maize. Using genetic line TB-10L18, our results indicate that nondisjunction of the B centromere occurs at an average frequency of 56.6%, based on four plants and 1306 pollen grains analyzed. This is consistent with the results of genetic studies using the same EA translocation. In addition, our results suggest that B chromosome nondisjunction can occur during the first microspore division. Spatial distribution of the B chromosome-specific probe appears to be largely confined to one tip of the sperm nucleus, and a DNA fragment found outside the pollen nuclei often hybridizes to the B chromosome-specific probe.

The B chromosomes of maize are extra, or supernumerary, chromosomes that occur in several races. They have no homology with the normal (A) chromosomes and are generally thought to be inert in that no major genes have been identified with them and they have no phenotypic effects on the plant except in high numbers (Carlson 1986). However, B chromosomes are known to modify crossing over frequencies of chromosomes in intact interphase nuclei within mature pollen of maize (Beckett 1991). The recent availability of a molecular probe directed to a unique centromeric DNA sequence within the B chromosome has allowed the detection of B chromosomes in interphase nuclei within intact cells (Alfenito and Birchler 1993; Shi et al. 1996).

Shi et al. (1996) demonstrated the feasibility of using the B chromosome-specific probe to identify B chromosome-containing sperm cells within mature pollen and pollen tubes. Here, we report the results of studies based upon four additional plants and on an improved technique for fluorescence in situ hybridization (FISH). We demonstrate that DNA-DNA hybridization procedures can be accurately used to determine frequencies of B chromosome nondisjunction during the second microspore division, as well as certain other aspects of B chromosome behavior during pollen development in maize.

MATERIALS AND METHODS

Plant material: Maize (Zea mays) genetic line TB-10L18 was grown in the greenhouse at Northern Arizona University from March through July 1996, under ambient light and humidity, with an average daytime temperature of 25°C and an average nighttime temperature of 18°C. This stock contains a B-A translocation derived from a break in the short arm of the B chromosome (BS) and a proximal break in the long arm of chromosome 10 (10L; Lin 1979). One of the two resulting chromosomes, 10-B, contains the centromere and short arm of chromosome 10 and a portion of BS. The reciprocal chromosome, B-10, consists of the centromere and long arm of the
B chromosome and most of 10L. Our stock was produced by crossing a male parent containing two B-10 chromosomes and two 10-B chromosomes with a female parent containing two normal 10 chromosomes, 10-N, and no B chromosomes. The construct TB-10L18 carries a dominant gene for anthocyanin (R-scm3) on 10L that pigments the aleurone of the endosperm and the scutellum of the embryo (LIN 1979; ALFENITO and BIRCHLER 1993). Because the seed parent was homozygous +g (colorless aleurone and scutellum), the B-10 chromosome can be tracked phenotypically.

When nondisjunction of the B-10 chromatids occurs during the second microspore division, one of the resulting sperm contains two B-10 chromosomes and one 10-B chromosome, whereas the other sperm contains only the 10-B chromosome. The construct TB-10L18 carries a dominant gene for anthocyanin (R-scm3) on 10L that pigments the aleurone of the endosperm and the scutellum of the embryo (LIN 1979; ALFENITO and BIRCHLER 1993). Because the seed parent was homozygous +g (colorless aleurone and scutellum), the B-10 chromosome can be tracked phenotypically.

For FISH experiments, fresh pollen was collected over a 2-hr period, fixed in a 95% ethanol-glacial acetic acid solution (3:1 v/v) for up to 24 hr, transferred to 70% ethanol and stored at ~20°, as described in more detail previously (Shi et al. 1996).

**DNA probe preparation:** The maize B chromosome-specific clone pZMBs was the same as that previously used (Stitt et al. 1996) and was generously supplied by JAMES A. BIRCHLER (University of Missouri, Columbia). T3 and T7 primers were used in the PCR to amplify the clone and incorporate biotin-16-dUTP. Miniagarose gel electrophoresis was used to verify that the PCR product was in the correct size range (1.1 kilobase pairs). The probe was cut with HaeIII restriction enzyme (Promega) and purified using the Qiagen PCR purification kit, resulting in two fractions of ~450 and 700 base pairs (bp).

**In situ hybridization:** Pollen was rinsed in water then incubated for 10 minutes at 75-79° in a hybridization mixture of 50% formamide, 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate), 10% dextran sulfate, 50 μg/ml herring sperm DNA, and 100-200 μg/ml DNA probe. Following denaturation, the pollen was incubated for 20-22 hr at 37° to allow hybridization of the DNAs. Controls included experiments in which the hybridization mixture did not contain the probe or in which the pollen was from the non-B-containing female tester described above.

**Detection and visualization of the probe:** After hybridization, pollen was rinsed three times at 44° in a solution of 50% denontized formamide and 2× SSC, followed by two rinses in PN buffer (0.1 M NaH2PO4, 0.1 M Na2HP04, 0.1% Nonidet P-40, pH 8.0) at 44°. The pollen grains were then incubated for 1 hr in the dark in blocking buffer (5%, w/v, Boehringer Mannheim blocking reagent, 0.1%, w/v, sodium azide in PN buffer) containing 3.5 μg/ml fluorescein isothiocyanate (FITC) avidin DCS (Vector Laboratories). Following three rinses in PN buffer at room temperature, the pollen was mounted in Vectashield antifade medium (Vector Laboratories) containing 0.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI).

**Observations were made with an Axioplan epifluorescence microscope (Carl Zeiss, Inc.) equipped with filters for visualizing DAPI and FITC fluorescence. Kodak Ektachrome 400 color slide film was used to record the images.**

**Image processing and enhancement:** Because DAPI and FITC fluorescences were detected using different filter sets, it was necessary to capture separate images of the labeled probe and the DAPI-stained nuclei. Thus, composite illustrations based on two images were made using a technique similar to that of MALUSZEŃSKA and HESLOP-HARRISON (1993). The original color slides were scanned into the computer (Dell OptiPlex GM+ 5135) using a ScanMaker 35t plus slide scanner (Microtek). The two images were then contrast adjusted and superimposed into a single image with the Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) imaging program. Processed images were recorded on Kodak Ektachrome 100 color slide film and a Polaroid CI-5000 digital palette film recorder, and then color prints were made from the slides.

**RESULTS**

Pollen from plants with two B-10 chromosomes consistently showed a FITC signal within some or all nuclei. The signal was quite localized as a bright, greenish-yellow spot (Figure 2, B, E and H), although some signals were brighter, larger, and/or more diffuse than others. Typically, only one signal was present in a given nucleus; however, occasionally two spots were seen in the sperm and/or vegetative nuclei. No FITC signals were found in pollen from plants without B chromo-

![Image of plant derivation](insert image here)

**Figure 1.**—Derivation of the plants of this study. 10-N, normal, intact chromosome 10. B-10, translocation between the short arm of the B chromosome and the long arm of chromosome 10 (10L); contains the centromere and long arm of the B chromosome, and most of 10L. 10-B, the reciprocal of B-10; consists of the short arm of the B chromosome, and the centromere and short arm of chromosome 10. VN, vegetative nucleus; GN, generative nucleus.
Figure 2.—Localizations of a B chromosome-specific probe within the mature pollen of maize (all ×425). (A) DAPI staining showing the two sperm nuclei (Sp) and the vegetative nucleus (VN). (B) Same pollen grain as in A, showing two FITC signals (arrowheads), which locate the B chromosome-specific probe. (C) Composite illustration based upon the original images of A and B, showing the location of the B chromosome-specific probe (arrowheads) within one of the sperm nuclei and the vegetative nucleus. (D) DAPI staining showing the two sperm nuclei (Sp) and the vegetative nucleus (VN). (E) Same pollen grain as in D, showing three FITC signals (arrowheads). (F) Composite illustration based upon the original images of D and E, showing the location of the B chromosome-specific probe (arrowheads) within each of the three pollen nuclei. (G) DAPI staining showing the two sperm nuclei (Sp), the vegetative nucleus (VN), and an extranuclear DNA fragment (Fg). (H) Same pollen grain as in G, showing two FITC signals. (I) Composite illustration based upon the original images of G and H, showing the location of the B chromosome-specific probe (arrowheads) within one sperm nucleus and the extranuclear DNA fragment. A FITC signal is also present within the vegetative nucleus, but does not show at this plane of focus.

somes nor when the FISH reactions were carried out without the B-specific probe being included in the hybridization mixture (data not shown).

Among the plants containing two B-10 chromosomes (four plants and 1306 pollen grains were examined), six classes of pollen grains were found with regard to the presence and location of the B chromosome-specific probe (Table 1). The most common was class A, which showed a signal in only one sperm nucleus and the vegetative nucleus (Figure 2, A–C). This pattern occurred at an average frequency of 56.6%. The next most common pattern of B-10 chromosome localization (class B) occurred, on average, in 37.2% of the pollen grains and showed a B probe signal in both sperm nuclei and the vegetative nucleus (Figure 2, D–F). Pollen grains with a B probe signal in the two sperm nuclei and not in the vegetative nucleus (class C) were found at an average rate of 1.7%, and pollen grains containing a signal only in one sperm nucleus (class D) were found at an average frequency of 1.6%. Pollen grains containing a FITC signal only in the vegetative nucleus (class E) were seen at an average rate of 0.6%, and pollen in which no signal was detected (class F) occurred at an average frequency of 2.3%.

Positionally within the sperm, the B probe signal was most often seen very close to one of the tips of the
Location of a B chromosome-specific probe within the nuclei of mature maize pollen

<table>
<thead>
<tr>
<th>Plant</th>
<th>Frequency of probe location in six pollen classes</th>
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<tbody>
<tr>
<td></td>
<td>A (1Sp and VN)</td>
</tr>
<tr>
<td>1 (n = 602)</td>
<td>70.3</td>
</tr>
<tr>
<td>2 (n = 224)</td>
<td>55.8</td>
</tr>
<tr>
<td>3 (n = 224)</td>
<td>64.3</td>
</tr>
<tr>
<td>4 (n = 256)</td>
<td>86.0</td>
</tr>
<tr>
<td>Average</td>
<td>56.6</td>
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</tbody>
</table>

n = number of pollen grains; 1Sp = one sperm; 2Sp = two sperm; VN = vegetative nucleus.

DISCUSSION

We have used a unique centric DNA sequence of the B chromosome of maize to carry out FISH procedures in mature pollen. By performing biotinylation during the PCR reaction, rather than using nick translation, we have significantly improved the signal strength of the FITC-labeled probe over that of a previous study (Losoff Rusche et al. 1996), without the need for amplification using antiavidin.

Our results are consistent with genetic studies on the frequency of nondisjunction of the B-10 chromatin during the second microspore division. Because the plants used for this study contained two B-10 chromosomes, the microspores resulting from meiosis should each contain one B-10 chromosome. Thus, pollen grains containing a B probe signal in one sperm nucleus and the vegetative nucleus (Figure 2, A–C; class A of Table 1) are the expected result of B-10 chromatin nondisjunction (Figure 3A). Ordinarily, only a single probe signal is seen in a given nucleus. We interpret the one signal in the sperm nucleus to actually represent both B centromeres that have remained very close together within the tightly packed chromatin of the sperm nucleus. The signal in the sperm nucleus is consistently larger and brighter than that in the vegetative nucleus. This pattern of probe location (in one sperm and the vegetative nucleus, class A of Table 1) was the most common, occurring on average at a rate of 56.6%. LIN (1979) found that the average rate of nondisjunction for the same translocation as used in this study, TB-10L18, was 52.6% in four sublines combined. These frequencies are lower than that of another translocation, TB-10L19, studied by LIN (1979), which showed nondisjunction at a frequency of 83%, and it is lower than that for other B-A translocations in general (Carlson 1986, 1988). LIN (1979) suggests that the break in TB-10L18, which is believed to be in the short arm of the B chromosome, may interfere with one of the components controlling nondisjunction.

The frequency of B chromatin nondisjunction in maize is quite variable. It is generally found to be between 50 and 100% among the various B-A translocations, and it can vary considerably from experiment to experiment (Carlson 1986, 1988). However, the level of nondisjunction appears to be particularly variable with TB-10L18 according to LIN (1979), who found a high degree of differences in nondisjunction frequencies both between (29.6–64.9%) and within sublines (30.2–60.4%). Such variability is in accordance with our results using FISH analysis, which showed nondis-
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A  ORIGIN OF CLASS A POLLEN

B  ORIGIN OF CLASS B POLLEN

C  ORIGIN OF CLASS C POLLEN

D  ORIGIN OF CLASS D POLLEN

E  ORIGIN OF CLASS E POLLEN

F  ORIGIN OF CLASS F POLLEN
junction from 36 to 70% among four plants investigated (Table 1). In our previous study, which used a single plant, we found the rate of nondisjunction to be 78.8% (Shi et al. 1996).

Pollen grains containing a B probe signal in each of the three pollen nuclei (Figure 2, D–F; class B of Table 1) are the expected result of normal B-10 chromatid disjunction during the first and second microspore divisions (Figure 3B). This pattern was found at an average rate of 37.2% (Table 1).

Three other patterns of probe location in mature pollen suggest that B-10 chromatid nondisjunction can also occur during the first microspore division at low frequencies. Pollen containing a probe in the two sperm nuclei and none in the vegetative nucleus was found at an average rate of 1.7% (range 0.8–2.7; class C of Table 1). This pattern could result from nondisjunction during the first microspore division, with the B-10 chromosomes going to the generative nucleus followed by normal disjunction during the second division. However, based on work using two B-A translocations in the same plant, Carlson (1969) concluded that the nondisjoined B chromosomes are distributed randomly to the two poles during the second microspore division. Thus, this pattern of probe location (class C) could also be the result of nondisjunction during both the first and second divisions, with one of two sets of nondisjoined B-10 chromosomes going to each of the sperm nuclei (Figure 3C). Similarly, the presence of a signal only within one sperm nucleus, which was found at an average frequency of 1.6% (range 1.3–2.0; class D of Table 1), is probably the result of nondisjunction during the first and second divisions as above, but with both sets of nondisjoined B-10 chromosomes going to the same sperm nucleus (Figure 3D). When such a sperm fertilizes the egg, the progeny would contain four B-10 chromosomes. Roman (1947) recognized that progeny containing four B chromosomes from a cross between a female with no B chromosomes and a male with two B chromosomes "cannot be accounted for on the basis of a single occurrence of nondisjunction." Randolph (1941) found this type of progeny at a frequency of 3.3%. The occurrence of nearly equal frequencies of pollen classes C and D is consistent with the interpretation of nondisjunction at the first division followed by random nondisjunction at the second division since half of the time the two sets of nondisjoined B chromosomes would be expected to go to different sperm and the other half of the time they would go to the same sperm nucleus. Equal frequencies suggest further that if there is nondisjunction at the first division then there will be nondisjunction at the second. Otherwise, the first pattern (class C) would represent a single event of nondisjunction and should be much greater than the second pattern (class D), which would require two nondisjunction events.

In classes C and D, half of the vegetative nuclei contain only the 10-B chromosome and not the B-10 (Figure 3, C and D). This condition would be expected to produce aborted or nonfunctional pollen due to a deficiency of 10L in the vegetative nucleus, which is responsible for normal pollen development (Kindiger et al. 1991). In our stock, meiosis produces two kinds of microspores: one type contains the B-10 chromosome plus a 10-N chromosome. The other type contains the B-10 chromosome and the 10-B chromosome (Figure 3, A–F). Both types of microspores are capable of undergoing B chromosome nondisjunction because the B-10 contains the B centromere and the entire long arm of the B, which are required to at least be in the same nucleus for nondisjunction to occur (Carlson 1986). If nondisjunction of the B-10 occurs during the first microspore division with the chromatids going to the generative nucleus (as in classes C and D), those microspores containing a 10-N chromosome would produce viable pollen. However, those microspores containing the 10-B would produce pollen with a vegetative nucleus containing only the 10-B, which is deficient for 10L. These pollen grains would, consequently, abort (Figure 3, C and D).

Thus, the frequencies of pollen in classes C and D of this study probably represent only half of the actual cases where nondisjunction occurred in both the first and second microspore divisions. Pollen abortion resulting from first division nondisjunction might explain a long-standing observation that hyperploid heterozygotes exhibit a level of pollen abortion above the normal level despite no predicted deficiencies from meiotic segregation (J. A. Birchler, personal communication).

In a small number of cases (average frequency of 0.6%), the B probe was found only in the vegetative nucleus (class E). This pattern could result from nondisjunction during the first division with the B-10 chromosomes going to the vegetative nucleus (Figure 3E). This type of pollen would be duplicate or triplicate for 10L and would likely not compete favorably for fertilization and, in any event, would be detected only with difficulty in genetic studies. Because this type of pollen occurs at a much lower frequency than that of classes C and D, it appears that, when nondisjunction occurs during the first microspore division, the B chromosomes are preferentially distributed to the generative nucleus. Such directed B chromosome nondisjunction during the first microspore division occurs routinely in rye (Muntzing 1946).

Class F pollen showed no B probe signal in any of the nuclei. This could be the result of probe labeling efficiency below the level of detection. Alternatively, it could be due to B chromosome loss during meiosis (Figure 3F). Because B chromosomes are often unpaired in meiosis, they may lag on the spindle and not be included in the daughter nuclei (Carlson and Roseman 1992). We found evidence for such B chromosome loss in the form of extranuclear, DAPI-staining, DNA...
fragments that hybridize to the B chromosome-specific probe (Figure 2, G–I). It is also possible that these chromatin fragments originated from B chromosome loss during pollen mitosis. In most cases when such a fragment is found, one sperm and the vegetative nucleus also contain the B probe. If the fragment resulted from partial nondisjunction during the second microspore division, with the lagging and loss of one of the B chromosomes, then the sperm containing the B probe would be euploid for the B-10 chromosome rather than hyperploid as would be expected from regular nondisjunction.

Some chromatin fragments do not hybridize to the B-specific probe. These fragments may represent knobbed A chromosome arms, the loss of which is known to be induced by B chromosomes, at least in a certain “high loss” line of maize (RHOADES and DEMPSEY 1973). More detailed studies of the extranuclear chromatin fragments are in progress.

The types of pollen found in classes C–F occur at quite low frequencies in the stock of this study. However, in certain lines, one or more of these pollen types may occur at significant levels. Therefore, studies on other B-A lines of maize, using the techniques of this investigation, are warranted.

Spatially within a sperm cell, the B-specific probe appears to be preferentially positioned very near one of the tips of the nucleus. We are currently quantifying this feature and will be making comparisons with the distribution of probes targeted to certain A-type DNA such as knobs, ribosomal DNA, and telomeres.

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