Sperm Identification in Maize by Fluorescence in Situ Hybridization

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The two sperm cells of common origin within the pollen tube of flowering plants are each involved in a fertilization event. It has long been recognized that preferential fusion of one sperm with the egg can occur in B chromosome–containing lines of maize. If the second pollen mitosis begins with a single B chromosome, nondisjunction will result in one sperm possessing two B chromosomes and the other containing no B chromosomes. The B chromosome–containing sperm most often fertilizes the egg, whereas the sperm nucleus with no B chromosomes fuses with the polar nuclei. Despite the obvious advantages of being able to recognize and then track, separate, and analyze one sperm type from the other, it has not been possible because of the lack of sufficient detectable differences between the two types of sperms. In this study, we used a B chromosome–specific DNA sequence (pZmBs) and in situ hybridization to identify and track the B chromosome–containing sperm cell within mature pollen and pollen tubes. Our results are consistent with conclusions from previous genetic studies related to B chromosome behavior during pollen formation. Within pollen tubes, the position in which the B chromosome–containing sperm travels (leading or trailing) in relation to the sperm cell lacking B chromosomes appears to be random.

INTRODUCTION

Each pollen grain of flowering plants produces two sperm cells, either before (tricellular pollen) or after (bicellular pollen) germination. Each sperm is involved in a fusion event: one unites with the egg to form the zygote, and the other unites with the central cell (containing the polar nuclei) to form the initial endosperm cell. This process is termed double fertilization.

Whether a specific sperm of the pair preferentially fertilizes the egg has long been debated (Maheshwari, 1950; Russell, 1985). In maize, it is known that double fertilization can be nonrandom in lines that contain B chromosomes, that is, supernumerary chromosomes that are not homologous to any of the normal set (A chromosomes). Because the B chromatids typically undergo nondisjunction during final pollen mitosis (division of the generative nucleus), the generative nuclei containing single B chromosomes result in one sperm that receives two B chromosomes and another that receives none (Roman, 1947). The sperm containing the B chromosomes most often (up to 75% of the time) fertilizes the egg (Roman, 1948).

Ultrastructural studies of maize pollen (McConchie et al., 1987; Rusche, 1988), pollen tubes (Rusche and Mogensen, 1988), and populations of isolated sperm cells (Wagner et al., 1989; Mogensen et al., 1990) have not revealed any consistent differences sufficient to distinguish one sperm type from

the other. Attempts to detect B chromosome–containing sperms in populations of isolated sperm cells by using a DNA-specific fluorochrome and flow cytometry have not been successful, due to the small amount of DNA contained in the B chromosomes compared with that in the A chromosomes (H.L. Mogensen, A. Chaboud, and C. Dumas, unpublished data). If the number of B chromosomes in the generative nucleus is increased, both sperms receive B chromosomes (Carlson, 1969).

In this study, we used a B chromosome–specific probe (Alfenito and Birchler, 1993) to determine which sperm of a pair contains the B chromosomes and thus is predisposed to fertilize the egg (Carlson, 1969). The application of such a technique opens new possibilities for the study of sperm cell biology and fertilization phenomena, including the recognition and collection of sperm populations enriched with male gametes that have a propensity to fertilize the egg, detection of differences in sperm-specific macromolecules between sperm types, determination of control mechanisms involved in gametic recognition and fusion in vitro, and tracking of sperm types within the pollen tube and during fertilization.

Here, we report results from fluorescence in situ hybridization (FISH) studies that have localized a B chromosome–specific DNA sequence within the sperm cells of maize in pollen and pollen tubes. The results are consistent with the interpretations of Roman (1947, 1948), which were based on genetic/cytogenetic studies, that B chromosome nondisjunction in the second pollen mitosis occurs at a high frequency. Our data also suggest that the order in which the sperms travel
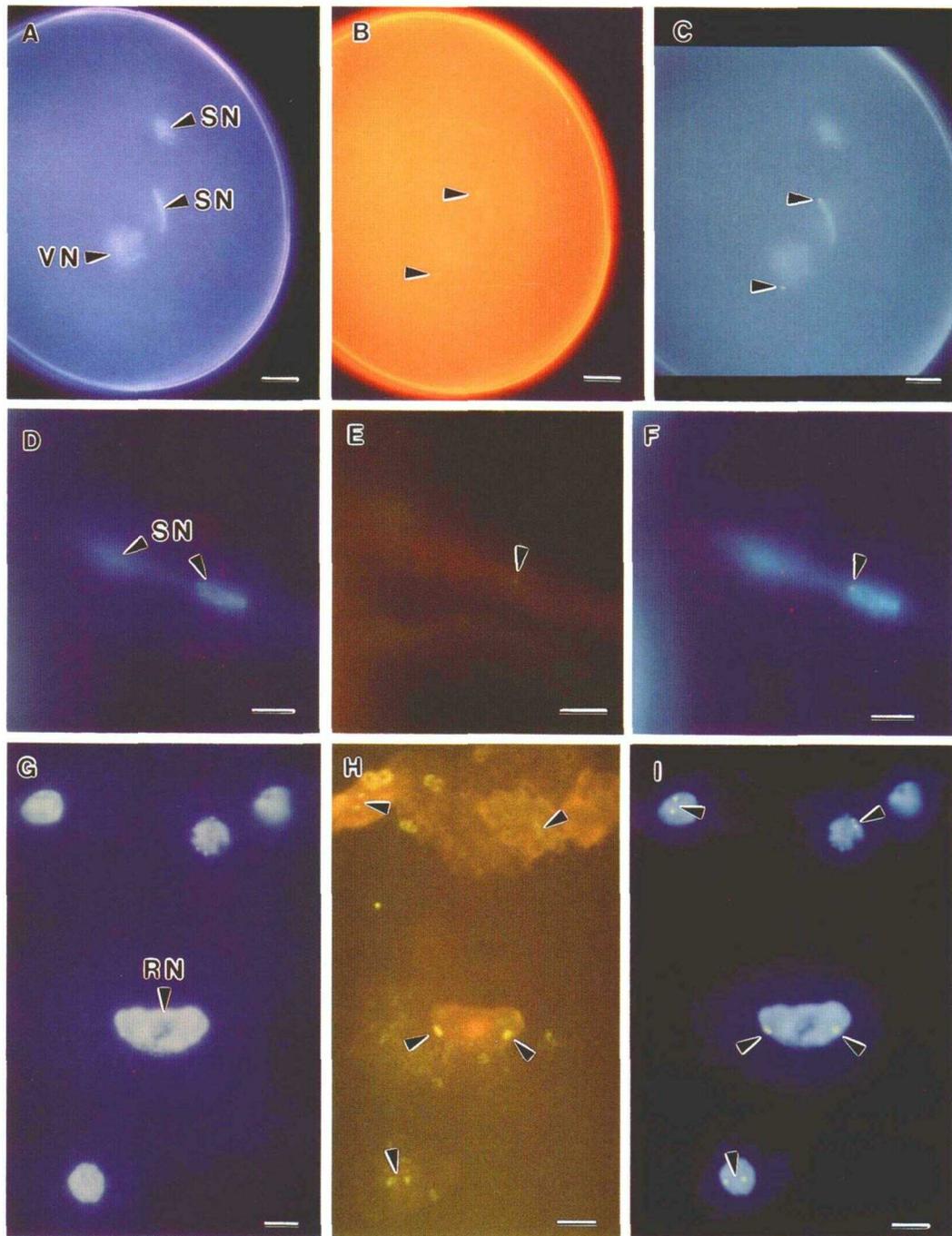
Figure 1. In Situ Hybridizations of a B Chromosome–Specific Probe (pZmBs) in the Pollen, Pollen Tube, and Root Tip of Maize.

(A) DAPI-stained pollen grain showing the two sperm nuclei (SN) and the vegetative nucleus (VN). Bar = 10 µm.

(B) Same pollen grain as in (A), showing two FITC signals (unlabeled arrowheads), which represent the locations of the B chromosome probe. Bar = 10 µm.

(C) Composite of the original images of (A) and (B), showing the localization of the B chromosome probe (yellow dots; arrowheads) in one sperm and the vegetative nucleus. Bar = 10 µm.

(D) DAPI-stained pollen tube showing two sperm nuclei (SN). Bar = 10 µm.

(E) Same pollen tube as in (D), showing an FITC signal (arrowhead). Bar = 10 µm.

(F) Composite of the original images of (D) and (E), showing the B chromosome probe (yellow dot; arrowhead) in one of the two sperm nuclei. Bar = 10 µm.

(G) DAPI-stained root tip nuclei (RN). Bar = 10 µm.

(H) Same nuclei as in (G), showing the FITC signals (arrowheads) within four of the five nuclei. Bar = 10 µm.

(I) Composite based on the original images of (G) and (H), showing the position of the B chromosome probes (yellow dots; arrowheads) within the somatic nuclei. Bar = 10 µm.

within the pollen tube is random and therefore may be unrelated to their fate in fertilization.

RESULTS

Distinct fluorescein isothiocyanate (FITC) signals, which indicate the locations of the B chromosome–specific probe, were obtained within nuclei of pollen, pollen tubes, and root tips from TB-10L18 plants, as illustrated in Figure 1. No signal was observed in control experiments in which the B chromosome probe was omitted or in somatic cells from root tips of cultivar Early Sunglow plants lacking B chromosomes (data not shown). Figure 1A shows the position of the 4′,6-diamidino-2-phenylindole (DAPI)–stained pollen nuclei within a pollen grain. Figure 1B shows two FITC signals within the same pollen grain. Figure 1C is an enhanced composite based on the original images of Figures 1A and 1B. In Figure 1C, it can be seen that the B chromosome probe is located within one sperm nucleus and the vegetative nucleus, which would be expected as the result of nondisjunction of the B chromatids in the second pollen mitosis, as illustrated in Figure 2A. Because the sperm nucleus contains only one signal, we presume that the two B chromosomes remain very close together after generative cell division and within the highly condensed sperm chromatin. Most often, the FITC signal within the sperm was located at one tip of the elongated nucleus. Upon careful examination and focus, it was apparent that the signal within the sperm nucleus was stronger than that in the vegetative nucleus; however, this difference in signal strength was not measured quantitatively.

Observations of 99 pollen grains revealed that the typical (78.8%) pattern of B chromosome probe localization was the same as that shown in Figures 1A to 1C, that is, one sperm and the vegetative nucleus contained the B chromosome probe. The next most common (10.1%) pattern showed an FITC signal within all three pollen nuclei, which would be expected as the result of normal disjunction of the B chromatids, as illustrated in Figure 2B. Three additional patterns were observed. Four percent of the pollen grains showed the presence of the B chromosome probe in both sperm nuclei but not in the vegetative nucleus. In 6.1% of the pollen grains, the signal was observed in only one sperm nucleus. In one pollen grain, a signal was seen only within the vegetative nucleus. The quantitative data are summarized in Figure 3.

In those pollen grains in which nondisjunction occurred in the second pollen mitosis, that is, the B chromosome probe was located in one sperm nucleus and the vegetative nucleus, no consistent pattern of sperm position was noted. The B chromosome–containing sperm was not consistently closer to or farther away from the vegetative nucleus.

Only pollen tubes fixed after 3 hr of in vitro growth contained sperm cells that had exited the pollen grain. In the 14 pollen tubes observed with an FITC signal, only one sperm nucleus of a pair contained the signal, as shown in Figures 1D to 1F.
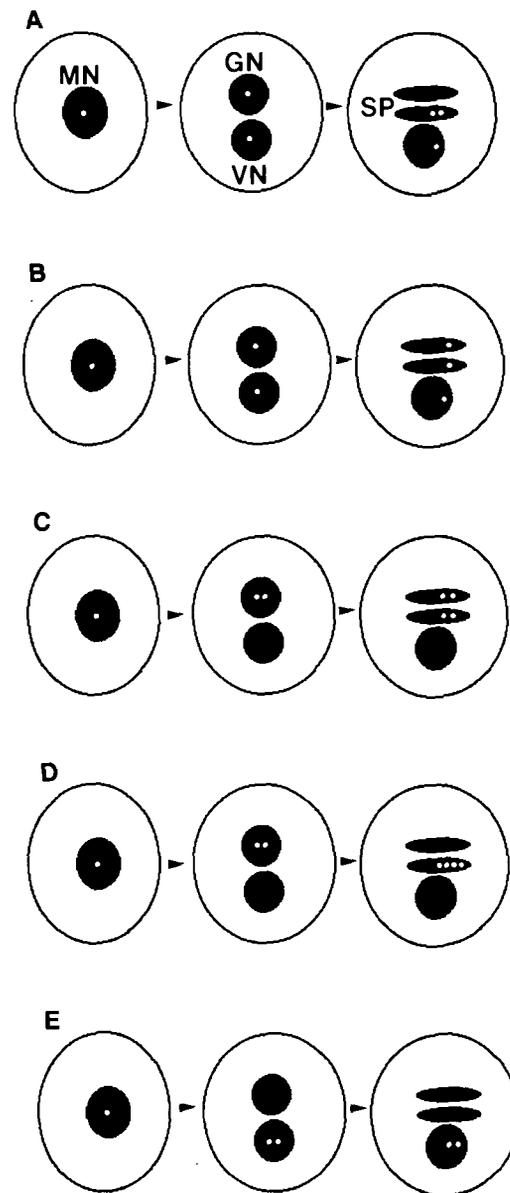
Figure 2. Possible Modes of B Chromosome Behavior during Pollen Development in Maize.

The B chromosomes are represented by white dots within the microspore nucleus (MN), the generative nucleus (GN), the vegetative nucleus (VN), and the sperm nuclei (SP).

(A) Nondisjunction of B chromatids during the second pollen mitosis.
(B) Normal disjunction of the B chromatids during both the first and second mitosis.
(C) Nondisjunction of the B chromatids during the first pollen mitosis. The B chromosomes go to the generative nucleus.
(D) Nondisjunction of the B chromatids during both the first and second pollen mitosis. The B chromosomes go to the generative nucleus.
(E) Nondisjunction of the B chromatids during the first pollen mitosis. The B chromosomes go to the vegetative nucleus.

Location of a B Chromosome Probe in Mature Maize Pollen
(n=99)
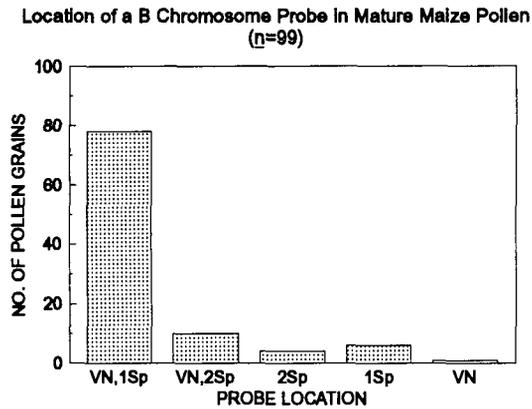
Figure 3. Quantitative Results of B Chromosome Probe Localization within Pollen Grain Nuclei.

The number of pollen grains (total of 99) containing the B chromosome probe within the vegetative nucleus (VN), one sperm nucleus (1Sp), and/or two sperm nuclei (2Sp) is depicted.

In 50% of the pollen tubes, the sperm containing the B chromosome probe was in the lead position, that is, distal to the pollen grain. In the other 50% of the pollen tubes, the sperm containing the B chromosome probe was proximal to the pollen grain. With regard to the sperm that contained the B chromosomes, no consistent orientation of the signal was found; that is, the tip of the nucleus containing the signal was not consistently closer to or farther away from the pollen grain.

FITC signals were particularly strong in root tip nuclei, as illustrated in Figures 1G to 1I. Unlike the condition found in pollen and pollen tube nuclei, the B chromosome signal was detected in two separate positions within somatic nuclei, sometimes at opposite sides of the nucleus, indicating that the B chromosomes are unassociated in root tip nuclei. Occasionally, the two signals could be seen to be composed of two subsignals each, as would be expected if DNA replication had been completed in preparation for mitosis. In a few somatic nuclei, a second, smaller set of two signals also was seen away from the larger set (data not shown).

DISCUSSION

The results of this study are in agreement with the findings of Alfenito and Birchler (1993) that the pZmBs clone contains a maize B chromosome–specific DNA sequence. FISH of this sequence in mature pollen showed FITC detection most often (78.8%) in only one sperm and the vegetative nucleus (Figure 3); two hybridization signals were typically found within each of the somatic root tip nuclei. These are the expected results from the experimental stock used in this study (Lin, 1979; Alfenito and Birchler, 1993), which was hyperploid heterozy-

gous for a B–A translocation (TB-10L18). No hybridization signals were detected in somatic nuclei of a maize line lacking B chromosomes.

Use of the pZmBs probe in this study allowed us to track B chromosomes within mature pollen directly. Due to various technical difficulties, cytological examination of B chromosome behavior during male gametogenesis has not been feasible (Roman, 1947; Carlson, 1986), although procedures are improving (Chang and Neuffer, 1994; Kindiger, 1994). Numerous genetic studies have shown that B chromosome nondisjunction occurs during the second pollen mitosis at a very high frequency—as high as 98% and rarely below 50% (Carlson, 1977). Normal disjunction of the B chromosomes during pollen development also has been demonstrated but at a relatively low frequency (Roman, 1947, 1948). Other possible modes of B chromosome behavior during pollen development have been proposed (Roman, 1947), but none has been definitively demonstrated.

The vast majority of the data from the present study using FISH analysis (Figures 1A to 1C, 2A, 2B, and 3) are in accordance with the genetic studies; however, other patterns of signal localization were observed (Figure 3): (1) the FITC signal was seen only in each of the two sperm nuclei; (2) the signal was present in only one sperm nucleus; and (3) only the vegetative nucleus contained the signal. Various scenarios of B chromosome behavior could explain these observed patterns of B chromosome probe localization, such as B chromatid nondisjunction during the first pollen mitosis, as illustrated in Figures 2C and 2E, and B chromatid nondisjunction during both the first and second pollen mitosis, as shown in Figure 2D. However, the results might be due to a slight variability in hybridization efficiency, and therefore, speculation regarding possible genetic consequences is not warranted here.

Roman (1948) offered two possible explanations for preferential fertilization of the egg by the sperm containing the B chromosomes. (1) The B chromosomes are directed to the sperm cell that is already preprogrammed to fuse with the egg. (2) The presence of the B chromosomes themselves confers upon the sperm cell containing them a propensity to fuse with the egg rather than with the central cell. Using two different B–A translocations in the same plant, Carlson (1969) provided evidence that the B chromosomes are not preferentially shunted into a particular sperm cell; thus, it appears that the B chromosomes somehow provide the sperm that contains them a competitive advantage to fertilize the egg. In this study, we did not find any positional pattern within the mature pollen grain that would indicate a particular sperm cell received the B chromosomes; thus, our data support those of Carlson (1969). However, at pollen maturity, the position of the sperms in relation to the vegetative nucleus is highly variable; therefore, any positional effects may have been lost by this stage. More meaningful data regarding this aspect of preferential fertilization would probably be obtained at earlier stages of sperm development.

Quantitative studies on the position of the vegetative nucleus relative to the generative cell (or sperm cells) within the pollen

tube show that the vegetative nucleus may be in the lead in 95% of tubes in *Brassica campestris* (Hough et al., 1984), in 70% of the tubes in *Helleborus foetidus* (Heslop-Harrison and Heslop-Harrison, 1986), and in 50% of the tubes in *Vallisnera spirallis* (Wylie, 1923). However, to our knowledge, whether the two sperms of flowering plants travel within the pollen tube in a specific order has not been addressed in any quantitative study. Presumably, a fixed order of sperm traveling within the pollen tube could be important to the process of double fertilization by affecting which sperm reaches the embryo sac first. In *Plumbago*, the sperms are highly dimorphic in size and cytoplasmic organelle content, and one is consistently closely associated with the vegetative nucleus. Ultrastructural studies of this plant, although not specifically addressing sperm order within the pollen tube, indicate that the larger sperm cell, which is associated with the vegetative nucleus, travels in the lead position; however, the number of observations is very limited (Russell and Cass, 1981). Similarly, in tobacco, the sperm cell associated with the vegetative nucleus appears to be in the lead position most often (Yu et al., 1992). Heslop-Harrison and Heslop-Harrison (1987) have observed in living pollen tubes of rye that the two sperms can pass each other while traveling within the pollen tube, indicating that sperm order is not consistent in this species.

Until now, it has not been possible to address sperm order within the pollen tubes of maize because of the lack of any sperm-specific markers. In this study, FISH analysis has provided such a marker. Although our results to date suggest that the sperms travel within the pollen tube in random order, the sample size is small. Moreover, whether sperm movement within pollen tubes grown in vivo is comparable to that within tubes grown in vitro is yet to be determined. Thus, the possible influence that sperm order within the pollen tube may have on preferential fertilization has not been fully delineated.

Further use of the pZmBs probe may make it possible to sort B chromosome–containing sperms from those that lack B chromosomes. The ability to tag the B chromosomes specifically with an FITC label should make it feasible to separate the two sperm types by using flow cytometry and cell sorting. The technology for isolating large numbers of maize sperm cells from mature pollen is already in place (reviewed in Dumas and Mogensen, 1993). Because the presence of the B chromosomes appears to render the sperm cell containing them more capable of fertilizing the egg (Carlson, 1969, 1986), it could be very useful to obtain populations of this sperm type separately from those without B chromosomes. Such sperm populations could be used for eliciting monoclonal antibodies and for identifying biochemical differences between sperm types that may help to identify the specific determinants involved in gametic recognition and fusion (Dumas et al., 1984; Chaboud and Perez, 1992; Dumas and Mogensen, 1993).

In vitro fertilization of isolated male and female gametes of maize also is now possible (Faure et al., 1994; Kranz and Lörz, 1994). Successful fertilization in vitro can be accomplished at a frequency of 80% (Faure et al., 1994). By using the pZmBs probe and appropriate genetic stock, it may be possible to compare fertilization success rates between in vivo and in vitro systems; that is, it may be possible to determine whether the sperm cell containing the B chromosomes retains its competitive edge for fertilizing the egg in vitro, as it does in vivo. Because the current detection procedures would render the sperm cells nonviable, it would be necessary to detect the pZmBs probe in the fusion products either by using FISH (on zygotes and early embryos) or DNA gel blot hybridizations (on older regenerated tissues). In vitro fertilizations involving the sperm and central cell could be similarly studied.

METHODS

Source of Pollen and Pollen Tubes

Maize (*Zea mays*) genetic line TB-10L18 was grown in the greenhouse at Northern Arizona University. This stock contains an interchange between the short arm of the B chromosome and the long arm of chromosome 10 (designated 10L-BL; Alfenito and Birchler, 1993). The stock was produced by a cross between a male parent containing two 10L-BL chromosomes and a female parent containing two intact 10 chromosomes and no B chromosomes. In the construct TB-10L18, the long arm of chromosome 10 carries a phenotypic marker (*R-scm3*) that pigments the aleurone of the endosperm and the scutellum of the embryo (Lin, 1979). Because the seed parent was homozygous *r-g*, it was possible to track the 10L-BL chromosome.

Due to nondisjunction of this chromosome during the second pollen mitosis (because it includes the centromere and distal tip of BL; Carlson, 1986), only one sperm receives the 10L-BL chromosomes. When this sperm fertilizes the egg, which is most often the case (Roman, 1948), the kernels can be selected easily by their purplish scutellum and nonpigmented endosperm (Lin, 1979; Alfenito and Birchler, 1993). Plants grown from these kernels (the plants in this study) are hyperploid heterozygotes, that is, they contain two 10L-BL chromosomes, the 10-B chromosome, and one intact chromosome 10. The 10L-BL chromosomes carry the *R-scm3* gene, whereas the intact chromosome 10 carries its recessive allele.

For in situ hybridization experiments, a tassel with its lower branches already shedding pollen was removed from the plant early in the morning and placed in a jar of water next to a sunny window in the laboratory. After vigorously shaking the tassel to remove any old pollen, we collected newly shed pollen on aluminum foil over a 2-hr period. The pollen was then placed into a covered Petri dish, held at 4°C for 4 to 6 hr, and then sprinkled onto microscope slides that had been coated with a thin layer of agar germination medium (Pfahler, 1967). After 1, 2, and 3 hr of culture, pollen and pollen tubes were fixed in an ethanol–glacial acetic acid solution (3:1 [v/v]) for 1 hr and then dehydrated in 95% (30 min) and absolute (1 hr) ethanol. The material was then air-dried and kept at 4°C until used for in situ hybridizations.

Source of Somatic Cells

Plants used for somatic cells, which served as controls, included both the TB-10L18 stock and a commercial line of sweet corn, Early Sunglow (D.V. Burrell Seed Co., Rocky Ford, CO), containing no B chromosomes. Seedlings were grown from kernels placed between moist filter paper in covered plastic containers at room temperature. Root tips, collected

from primary roots that had grown ~5 cm, were fixed in methanol–acetic acid (3:1 [v/v]) for 24 hr. Fixed root tips were then processed according to the procedures of Zhu et al. (1995) and Shi et al. (1996). After digestion with 0.2% cellulase and 2% pectolyase for 1.5 to 2.5 hr to remove the cell wall, the material was treated for 10 min in 0.075 M KCl and then postfixed in the same fixative. Protoplasts were centrifuged, resuspended in 70% ethanol, and dropped from ~30 cm onto microscope slides coated with Vectorbond (Vector Labs, Inc., Burlingame, CA). After flame drying, the material was stored at −70°C until use.

DNA Probe Preparation

The maize B chromosome–specific clone, pZmBs, previously identified and characterized by Alfenito and Birchler (1993), was used for the in situ hybridization studies. This clone, which contains a 1.1-kb BamHI-AccI fragment in the pBluescript SK+ (Stratagene) vector, was supplied to us by J.A. Birchler (University of Missouri, Columbia). Insert fragments were amplified by polymerase chain reaction (PCR), using T3 and T7 primers. The PCR products were examined by miniagarose gel electrophoresis for size verification and purified by Wizard PCR Preps (Promega DNA purification system). The length of the amplified probe was ~1200 bp, including a 50-bp sequence on the flanks of the plasmid vector.

To facilitate probe penetration of the pollen and pollen tube wall, the purified PCR product of the pZmBs insert fragment was labeled by nick translation with biotin-14-dATP by using the BioNick labeling system (Gibco BRL, Gaithersburg, MD), according to the manufacturer's specifications. The labeled probe ranged in length from 50 to 500 bp.

In Situ Hybridization

Pollen and pollen tubes with agar growth medium were rehydrated and collected into a 1.5-mL Eppendorf tube. Materials were incubated in 70% formamide, 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 5 min at 70°C and then quickly dehydrated through an ethanol series (70%, 90%, and absolute) at −20°C. Before each change, brief centrifugations were made to prevent loss of specimens. The hybridization solution (50% formamide, 2 × SSC, 10% dextran sulfate, and 50 μg/mL biotinylated B chromosome sequence) was denatured at 70°C for 5 min and then added to the Eppendorf tube containing the pollen and pollen tubes. Hybridization was allowed to proceed overnight at 37°C. Control experiments were performed as given above, except that the hybridization solution did not contain the biotinylated B chromosome sequence.

Somatic root tip protoplasts were processed similarly, except that they were left attached to the slides. Three experiments using complete hybridization medium were performed on pollen and pollen tubes. Two control experiments were run using hybridization solution without the B chromosome probe. Experiments on root tip cells from plants without B chromosomes were performed twice.

Detection and Visualization of the Probe

Following hybridization, specimens were rinsed with 50% formamide and 2 × SSC in PN buffer (0.1 M NaH$_2$PO$_4$, 0.1 M Na$_2$HPO$_4$, and 0.1% Nonidet P-40, pH 8.0) at 42°C. Materials were then incubated in PNM buffer (5% nonfat dry milk and 0.1% sodium azide in PN buffer) with 5 μg/mL fluorescein isothiocyanate (FITC) avidin DCS for 1 hr, rinsed

in PN buffer, and then mounted in Vectashield antifade medium (Vector) containing 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI).

Observations were made with an Axioplan epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with excitation and emission filters appropriate for visualizing FITC and DAPI. Images were recorded with Kodak Ektachrome 400 color slide film.

Image Processing and Enhancement

Because FITC and DAPI fluorescence are detected by using different filter sets, it was necessary to capture separate images of the FITC-labeled pZmBs probe and the DAPI-stained nuclei. Thus, composite illustrations based on both images were made using a technique similar to that of Maluszynska and Heslop-Harrison (1993). The original color slides were scanned into a Macintosh computer (Apple, Cupertino, CA) by using a scanner (model ES-600C; Epson, Torrance, CA). The two images were then contrast-adjusted and superimposed into a single image with the Photoshop (Adobe Systems Inc., Mountain View, CA) imaging program. Processed images were recorded on Kodak Ektachrome 100 color slide film by using a Polaroid CI-5000 digital palette film recorder.

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