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Fluorescent *in situ* hybridization to soybean metaphase chromosomes

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Abstract

Repetitive DNA sequences were detected directly on somatic metaphase chromosome spreads from soybean root tips using fluorescent *in situ* hybridization. Methods to spread the forty small metaphase chromosomes substantially free of cellular material were developed using protoplasts. The specific DNA probe was a 1.05 kb internal fragment of a soybean gene encoding the 18S ribosomal RNA subunit. Two methods of incorporating biotin residues into the probe were compared and detection was accomplished with fluorescein-labeled avidin. The rDNA probe exhibits distinct yellow fluorescent signals on only two of the forty metaphase chromosomes that have been counterstained with propidium iodide. This result agrees with our previous analyses of soybean pachytene chromosomes [27] showing that only chromosome 13 is closely associated with the nucleolus organizer region. Fluorescent *in situ* hybridization with the rDNA probe was detected on three of the forty-one metaphase chromosomes in plants that are trisomic for chromosome 13.

Introduction

In situ hybridization techniques developed in recent years are an important tool for the detection of specific nucleic acid sequences directly within cells or on chromosomes. Initially, *in situ* hybridization was used for the localization of DNA sequences on *Drosophila* polytene chromosomes or highly repeated sequences on metaphase chromosomes of diploid cells. In the past ten years, numerous refinements of the detection procedure have made possible the rapid, sensitive detection of short unique DNA sequences directly on mammalian metaphase chromosomes [2, 4, 13, 15, 16, 31]. *In situ* hybridization is a viable method for investigation of the spatial order of genes in interphase nuclei [15], the mechanism of gene amplification [30], and the identification of foreign DNA (transformed or viral) within animal and plant cells [8, 14].

The use of *in situ* hybridization in plants lags considerably behind its applications in cytogenetics of human and other animal systems. A major factor contributing to the difficulty in plants is obtaining mitotic and meiotic chromosomes free of cell wall material. Hybridization to plant metaphase chromosomes is often impeded due to their highly condensed nature. Difficulties are also encountered in the chromosome karyotyping of many plants including soybean, *Glycine max*. Soybean contains $2n = 40$ small (1.2–2.84 μm) morphologically similar mitotic metaphase chromosomes [25]. Despite its considerable economic importance, soybean lacks a cytogenetic map and a karyotype of all 20 soybean chromosomes has only recently been constructed for the relatively

uncondensed pachytene chromosomes [27]. In this report, we demonstrate the first application of *in situ* hybridization directly to individual soybean metaphase chromosomes.

Materials and methods

Plant material

Root tips from *Glycine max* (L) Merr. cv. Williams were used as the source for metaphase chromosomes. Seed of the primary trisomic S (satellited chromosome 13) line in soybean cv. Hodgson [24] were obtained from Reid Palmer, USDA/ARS, Agronomy Department, Iowa State University.

Chromosome preparation

Root tips were collected from secondary roots of seven-day-old seedlings grown in a sandbox in a greenhouse and pretreated for 2 to 4 h in 0.05% 8-hydroxyquinoline at 15 °C in order to obtain cells with a higher mitotic index. Root tips were then fixed in freshly prepared methanol, acetic acid, and chloroform (3 : 1 : 1), stored at − 20 °C, and used within 1 to 2 weeks for optimal results. Root tips were washed thoroughly in 0.01 M sodium citrate-citric acid (SC-CA) buffer at pH 4.6. Cell walls were digested in 2% cellulase ('Onozuka' R10) and 1% pectinase (Sigma) in SC-CA buffer for 2 h at 37 °C. Root tips were aspirated several times through a siliconized pasteur pipette to facilitate digestion of cell wall material. After enzymatic treatment, cells were subjected to a hypotonic treatment in 75 mM KCl for 10 min. A final fixation in the above fixative was performed for one hour on ice. Cells were dropped from a pasteur pipette onto methanol-cleaned and cold (− 70 °C) slides to promote chromosome spreading and were dried overnight at room temperature. Prepared slides were stored at − 70 °C in a vacuum-sealed bag and used within one week.

Probe and labeling methods

The soybean 18S rDNA probe pSR1.2B3 [3] contains a 1.05 kb internal fragment of the full 1807 bp rDNA gene and was supplied by R. Meagher, University of Georgia, Athens. Probe DNA was directly labeled by nick translation with bio-11-dUTP (Enzo Biochemical, New York) as per instructions from the supplier, resulting in incorporation levels of 30 to 50% bio-11-dUTP. Probe fragment size was controlled by adjusting the concentration of DNAse in the nick translation reaction to yield fragments between 300 to 1000 nucleotides. Fragment size was determined using alkaline gel electrophoresis followed by transfer to nitrocellulose and was visualized with strepavidin-conjugated alkaline phosphatase (Bethesda Research Laboratories). An indirect labeling method consisting of poly(T)-tailing DNAse-digested probe DNA was also employed. Detection of poly(T)-tailed probe was accomplished with a biotin-labeled poly(dA) molecule (BioBridge, from Enzo Biochemical, New York).

In situ hybridization

In situ hybridization was performed as described by Lawrence *et al.* [15] with modifications. Slides were treated with 100 μg/ml RNAse A (Sigma) in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 30 min at 37 °C and then sequentially dehydrated in an ethanol series of 70%, 95%, and 100% ethanol at room temperature. Slides were incubated in 0.1 M triethanolamine, pH 8.0, and 0.25% acetic anhydride for 10 min followed by denaturation in 70% formamide, 2 × SSC, pH 7.0, at 70 °C for 150 s and then immediately dehydrated 5 min each in 70%, 95%, and 100% ethanol at − 20 °C. They were then treated with 100 to 300 ng/ml proteinase K in 20 mM Tris-HC, 2 mM $CaCl_2$, pH 7.5, at 37 °C for 5 to 7.5 min and dehydrated as above. Probe and calf thymus DNA were denatured at 95 °C for 10 min, quick-cooled on ice, and then added to a hybridization mix. The final concentrations in the mix were 50% formamide,

2 × SSC, 10% dextran sulfate, 500 μg/ml calf thymus DNA, and 1 μg/ml denatured probe DNA. Of this mix 20 μl was applied to each slide under a 22 mm × 30 mm glass coverslip and sealed with rubber cement. Slides were incubated for 3 to 6 h in a humid chamber at 37 °C. After hybridization, slides were washed 2 times, 10 min each, in 50% formamide, 2 × SSC, pH 7, at 42 °C; 2 times, 5 min each, in 2 × SSC, pH 7, at 42 °C; and once in 1 × SSC, pH 7, for 10 min at room temperature. Slides were placed in PN buffer (0.1 M sodium phosphate, 0.05% Nonidet P-40, pH 8). Slides were not allowed to dry after this point. When poly(T)-tailed probe was used, 35 μl of a 1 : 50 dilution of the *BioBridge* labeling molecule in PN buffer was applied to each slide, covered with a parafilm coverslip, and incubated 10 min at room temperature. Slides were washed 3 times, 10 min each, in PN buffer at 42 °C with gentle agitation.

Detection and microscopy

The next steps in the detection procedure follow the protocol of Pinkel *et al.* [20] with modifications. Slides were incubated for 5 min in PNM buffer [PN buffer with 5% nonfat dry milk (Carnation)] and then briefly drained. Each slide was layered with 35 μl of 3 μg/ml fluorescein avidin DN (Vector Research Laboratories) in PNM buffer, covered with a parafilm coverslip, and incubated for 20 min at 37 °C. Slides were washed with buffer 3 times for 5 min each at 42 °C. Slides were incubated in 5% normal goat serum in PN buffer, 35 μl per slide, for 5 min at room temperature and then drained. To each, 35 μl of 5 μg/ml biotinylated goat anti-avidin antibody (Vector Research Laboratories) in PN buffer was applied and incubated 20 min at room temperature. Slides were washed as above, and a final layer of fluorescein avidin DN was applied. After washing, slides were mounted in 0.4 μg/ml propidium iodide in an anti-fade solution [11].

Color slides of metaphase plates were taken using a Reichert-Jung Polyvar microscope equipped with a Reichert Plan Apo oil immersion objective (100 ×, 1.32 numerical aperture) using Kodak Ektachrome 400 slide film with exposure times of 120 to 150 s. A B1 epifluorescent module with the following filters was used for simultaneous visualization of propidium iodide-counterstained chromosomes and fluorescein signal: excitation, 450 to 495 nm; dichroic mirror, DS 510 nm; emission, LP 520 nm. A B4 module was used for visualization of the fluorescein signal alone and has the following filters: excitation, 475 to 495 nm; dichroic mirror, DS 510 nm; emission, BP 520 to 560 nm. A G1 module allowed visualization of propidium iodide stained chromosomes without the fluorescein signal: excitation, 546 nm; dichroic mirror, DS 580 nm; emission, LP 590 nm.

Results

Chromosome spreading for in situ *hybridization*

The overall approach for preparation of metaphase chromosomes of sufficient quality for fluorescent *in situ* hybridization involved three major steps including arrest of cells at mitotic metaphase by chemical pretreatment, digestion of cell walls to obtain a relatively clean population of protoplasts, and spreading the chromosomes by lysing protoplasts directly on the slides. The chromosomes were detected by fluorescent dyes such as propidium iodide. *In situ* hybridization was performed using a biotin-labeled soybean ribosomal DNA (rDNA) probe and the regions of homology were visualized using an avidin-fluorescein conjugate similar to procedures for human chromosomes [15, 20].

Metaphase chromosomes free of cellular debris were obtained following modifications of existing methods used previously for *Zea mays* [23], *Sinapis alba* [6], *Crepis capillaris* [1], *Apium graveolens* and *Brassica carinata* [18], and *Triticum monococcum* and *Papaver somniferum* [9]. In general, cellular debris must be removed by enzymatic digestion of the mitotic tissue and numerous washes of the protoplasts before they are spread directly on slides. Initial attempts with

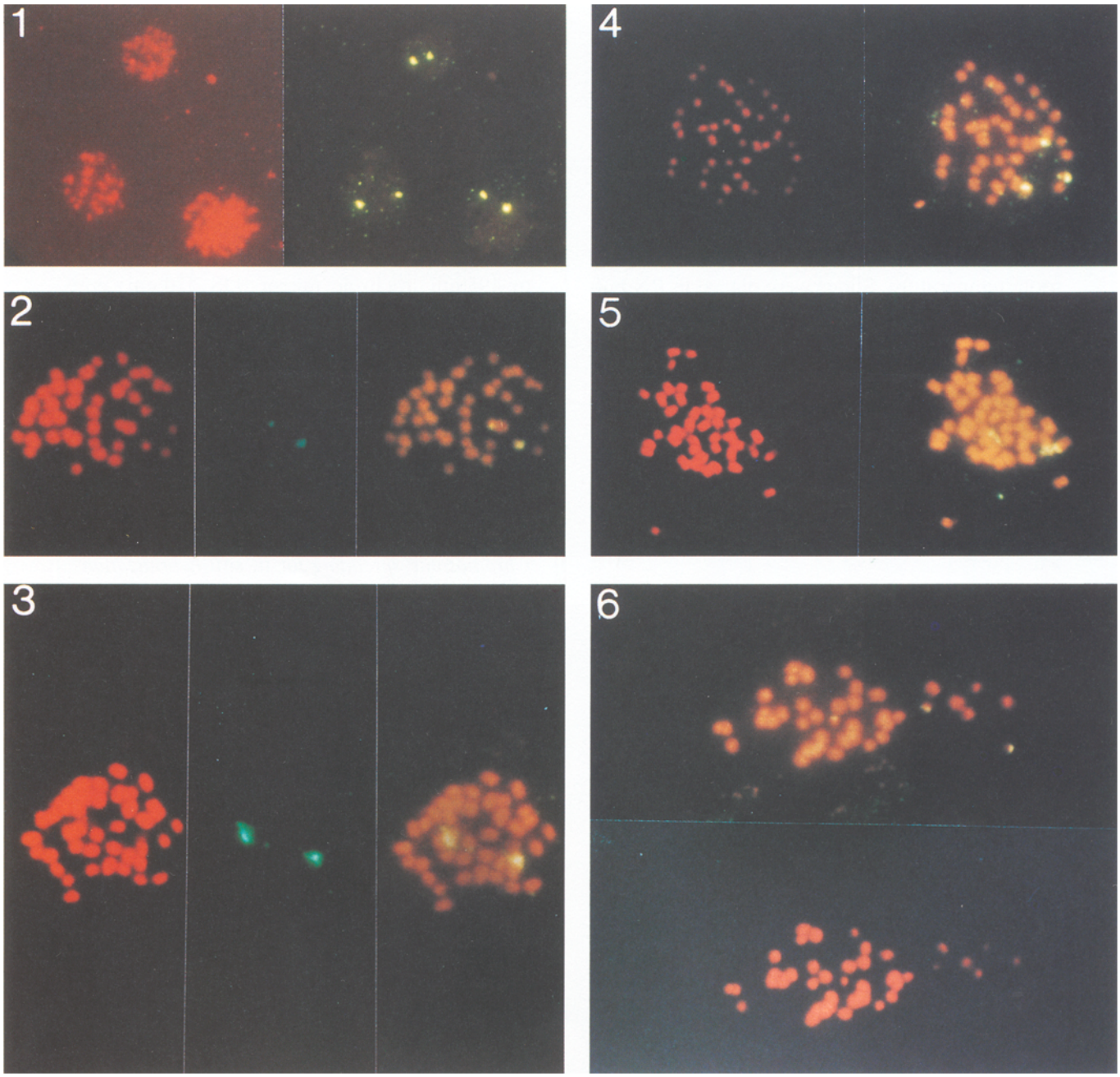

Fig. 1. Fluorescent detection of rDNA within interphase or clumped metaphase chromosomes from soybean root tips. The nick-translated and poly(T)-tailed pSR1.2B3 probe was used. Left, propidium iodide staining of total DNA; right, two very strong yellow fluorescent signals are observed in each of the three cells the B4 filter that detects only the fluorescein label. Magnification × 1200.

Fig. 2. Detection of rDNA genes on soybean metaphase chromosomes. Bio-11-dUTP was incorporated into the pSR1.2B3 probe using nick translation without subsequent poly(T)-tailing. Chromosomes are counterstained with 0.25 μg/ml propidium iodide. Left panel, detection of propidium iodide staining with the G1 filter; middle, bluish fluorescence of the fluorescein signal only using the B4 filter that blocks propidium emission; right, yellow fluoresence of the fluorescein signal on the chromosomes using the B1 filter combination. Magnification × 1200.

soybean yielded slides relatively free of cellular debris but resulted in a high proportion of chromosomes in a 'clumped' arrangement as shown in Fig. 1. Although *in situ* hybridization can be performed on these clumps yielding two distinct fluorescent signals with the rDNA probe (Fig. 1, right), the metaphase chromosomes in this arrangement offer no advantages over interphase cells.

Failure of the soybean chromosomes to spread could result from the pretreatment used for arresting cells, the fixative, or the way slides are prepared. Initially, we used colchicine pretreatment, Farmer's fixative (3 parts ethanol, 1 part acetic acid), and room temperature spreading. We then explored alternatives in an attempt to obtain a higher proportion of well spread chromosomes. Other pretreatment agents used to arrest metaphase cells are para-dichlorobenzene, α-monobromonaphthalene, or 8-hydroxyquinoline. As detailed in Materials and methods, the most promising results were obtained with 8-hydroxyquinoline followed by tissue fixation in methanol-acetic acidchloroform (3 : 1 : 1). Humidity and cold slides sometimes aid chromosome spreading. Best results with soybean chromosomes were obtained when methanol cleaned slides were stored at − 70 °C for several hours before spreading and the prepared cell suspension was dropped immediately onto frosted slides taken from the freezer. Using this combination of pretreatment, fixative, and slide preparation, 50 to 75% of the soybean metaphase cells exhibit 40 well spread chromosomes that allowed direct detection of the *in situ* signal to individual chromosomes as shown in Figs. 2–6.

Comparison of probe labeling methods for in situ hybridization

Nick translation was used to incorporate the bio-11-dUTP into total plasmid DNA and the size of fragments are adjusted to approximately 300–1000 nucleotides. The nick-translated fragments were used directly as a probe or were further labeled by addition of poly(T) tracts to the fragment ends with terminal deoxynucleotide transferase and hybridization to a poly(dA) molecule containing incorporated biotins (BioBridge). Thus, additional biotin residues should increase the amount of label available for detection. This appeared to be the case from comparing the intensity of the nick-translated probe to that of the nick-translated and tailed probe as judged by alkaline gel electrophoresis and detection on gel blots using avidin-alkaline phosphatase (data not shown).

Figures 2 and 3 compare the results of *in situ* hybridization with the two labeling protocols. The rDNA probe was nick-translated with bio-11-dUTP (Fig. 2) or was nick-translated and also poly(T)-tailed (Fig. 3) to incorporate more biotin residues before use as a probe. In each case, two distinct yellow fluorescent signals are visible on separate metaphase chromosomes. However, when signal intensities are compared, it can be seen that signal from the probe that has also been

◀ *Fig. 3.* Enhancement of *in situ* hybridization signal using additional biotin incorporation. Bio-11-dUTP was incorporated into the rDNA probe by nick translation followed by subsequent poly(T)-tailing and hybridization with a poly(dA) : biotin molecule as described in Materials and methods. Left panel, propidium iodide staining of chromosomes; middle, fluorescein signal only; right, simultaneous visualization of the fluorescein and propidium stains.

Figs. 4 and 5. Detection of rDNA sequences on soybean metaphase chromosomes from plants trisomic for chromosome 13. The rDNA probe was labeled by both nick translation and poly(T)-tailing. Left, propidium iodide staining showing 41 chromosomes; right, simultaneous visualization of chromosomes and the fluorescein signal. Three intense yellow fluorescent fluorescein signals are distinguishable in each figure. Magnification × 1200.

Fig. 6. In situ hybridization to a trisomic plant using a nick-translated rDNA probe without subsequent poly(T)-tailing. Top, fluorescein signal on three of the metaphase chromosomes as compared to the propidium iodide stained chromosomes (bottom). Magnification × 1200.

poly(T)-tailed is several-fold more intense than the signal with a probe that is only nick-translated. This is especially evident when the figures that show the fluorescein signals alone are compared (Figs. 2 and 3, middle panels in which the fluorescein is bluish green using this excitation and barrier filter combination). In more than 95% of the chromosome spreads examined, it was apparent that there were two signals for the rDNA probe that were definitely located on individual chromosomes as opposed to background signal.

Analysis of trisomic plants by in situ *hybridization*

A primary trisomic line for the satellited chromosome 13 that associates with the nucleolus [24, 28, 29] was examined. Using the 18S rDNA probe, three very strong fluorescent signals were routinely visualized on individual metaphase chromosomes from plants trisomic for the satellited chromosome as shown in several independent experiments (Figs. 4–6). Unambiguous assignment of the signal was aided by use of filter combinations that allow visualization of the same chromosome spread with either the propidium iodide alone or with the fluorescein signal superimposed on the propidium iodide-stained chromosomes. For example, two adjacent chromosomes in Fig. 5 display signal along with a third separate one. Use of biotinylated goat anti-avidin antibody to amplify the signal generally leads to a higher amount of background fluorescent spots and this step can be omitted for a cleaner preparation. Again, the probes prepared using nick translation and poly(T) additions (Figs. 4 and 5) gave a more intense signal than those with nick translation alone (Fig. 6).

Discussion

The application of nonradioactive *in situ* hybridization has facilitated the mapping of ribosomal RNA genes in several plant genomes [17, 21, 32]. Other repeated DNA sequences have been used as cytological markers in conjunction with nonradioactive *in situ* hybridization in wheat [22] and a low-copy DNA sequence in rye coding for the endosperm storage protein gene, *Sec-1*, has been localized to chromosome 1RS [7]. With few exceptions, *in situ* hybridizations to plant chromosomes have utilized either tritium or nonradioactive, enzymatic detection using biotin-labeled probes. In contrast, fluorescent detection is currently used extensively in human chromosome analysis. Our results and those of Maluszynska and Schweizer [17] demonstrate the feasibility of employing fluorescent detection methods with plant chromosomes using probes to repetitive DNA.

In most of the plant species cited above, the metaphase chromosomes can be karyotyped by size or differential staining. In soybean, however, the 40 metaphase chromosomes are very small. They cannot be karyotyped by size and do not produce significant banding patterns for identification [12, 25]. In order to identify the individual chromosomes, meiotic cells must be employed and the first karyotype of soybean pachytene chromosomes was recently reported [27]. Each individual chromosome was identified by length and its heterochromatic and euchromatic regions. A considerably large segment of the chromosome 13 short arm was associated with the nucleolus in pachytene and often appeared attached but the satellited nucleolar organizing region cannot be recognized in contracted somatic metaphase chromosomes. The 18S and 25S rRNA genes are on the chromosomes that associate with the nucleolus in most organisms. Four primary trisomics of soybean have been identified by pachytene analysis and include chromosomes 1, 4, 5, and 13 [28]. We observed *in situ* hybridization signals on three individual chromosomes of plants trisomic for chromosome 13 (Figs. 4–6). The intense signal from the rDNA probe appears to cover much of the chromosome so that the individual chromatid halves of the mitotic metaphase chromosomes are not distinguished. If the 18S rDNA genes were present on chromosomes other than chromosome 13, fluorescent signal should be observed on additional chromosomes.

We consistently observed only two or three hybridization signals in disomic and trisomic spreads, respectively, that had low background fluorescence. Thus, the 18S rDNA genes appear to be only on chromosome 13 as defined by the limits of sensitivity of this *in situ* hybridization procedure. This agrees with the observation that only chromosome 13 is associated with the nucleolus during pachytene. Although soybean is likely an ancient polyploid, it appears that rDNA genes have been lost by mechanisms that lead to diploidization of many sequences during evolution [29].

Skorupsa *et al.* [29] also detected two and three signals respectively in 'squashes' of interphase cells from soybean disomic and trisomic lines using peroxidase detection of a biotin-labeled maize rDNA probe. However, the signal was not visualized directly on the chromosomes because of difficulties in spreading chromosomes that would survive the *in situ* hybridization conditions. In the present report, we have developed reliable methods for spreading the 40 mitotic soybean chromosomes and have used fluorescent labeling to simultaneously stain the chromosomes and detect the hybridization target sequences.

Further refinements in the *in situ* hybridization technique for soybean will be to localize non-repetitive cloned genes to a particular chromosome. Meiotic karyotyping [27] is currently performed with a 'squash' technique without removal of cell walls which may leave too much debris for *in situ* hybridization to be effective with low background signal. Additionally, it is rare to obtain chromosome spreads that display all 20 meiotic chromosomes well separated within a chromosome spread of an individual cell. *In situ* localization using mitotic metaphase cells from aneuploid lines as demonstrated in this report is a potential alternative. In this regard, we are currently examining the progeny of wide species crosses in an effort to generate and identify alien addition and substitution lines.

Improvements in sensitivity must be achieved to allow rapid and reliable detection of short, non-repetitive probes using pachytene or metaphase soybean chromosomes. There are very few reports of low-copy detection in plants to date and most of these have employed a statistical analysis to verify the presence of the label consistently on a particular chromosome. These include detection of the *waxy* gene on 48% of the chromosome 9 maize pachytene spreads that were examined using tritium detection [26]; tritium detection of the parsley chalcone synthase gene [10]; detection of integrated T-DNA in transformed *Crepis capillaris* [1]; and detection of the low-copy *Sec1* storage protein in rye [7]. Alternatively, it has been found that increasing the target size around a single-copy gene will allow consistent chromosome localizations despite dispersed repeats present in human chromosomes. Thus, overlapping cosmid clones or yeast artificial chromosomes containing large (greater than 100 kb) inserts can be used to effectively hybridize more biotin residues to the target region [30, 19]. The dispersed chromosomal repeats do not interfere or can be suppressed with unlabeled chromosomal DNA before hybridization.

We can roughly calculate the target size of the ribosomal probe used in the present studies in order to estimate the size of a contiguous probe that might be needed to effect a signal for a single-copy gene on the soybean metaphase chromosomes with our current technology. The 18S and 25S rDNA gene unit in soybean has a 7.8 kb total cistronic length and is present in about 500 to 800 copies per haploid genome [3, 5]. Assuming 800 copies and 100% hybridization of the 1.05 kb probe to each of the repeat units, then our signal detected a total of 800 kb over a 6200 kb tandem chromosomal region. A 10-fold reduction in signal intensity should be reliably detectable without computer enhancement based on the very intense signal seen in Figs. 2–6. Thus, an 80 kb minimum target area should suffice. It is not unreasonable to obtain 150–300 kb inserts using yeast artificial cloning technology. In summary, several factors including increased clone size, improvements in spreading pachytene chromosomes, and increased sensitivity for low light levels will be needed to refine the fluorescent *in situ* hybridization process in soybean and other plant species carrying small chromosomes.

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