Chromosome painting in plants: *In situ* hybridization with a DNA probe from a specific microdissected chromosome arm of common wheat

(chromosome-specific sequences/microdissection/degenerate oligonucleotide-primed PCR/*Triticum aestivum*/genome evolution)

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ABSTRACT   We report here on the successful painting of a specific plant chromosome within its own genome. Isochromosomes for the long arm of chromosome 5 of the wheat B genome (5BL) were microdissected from first meiotic metaphase spreads of a monoisosomic 5BL line of the common wheat *Triticum aestivum* cv. Chinese Spring. The dissected isochromosomes were amplified by degenerate oligonucleotide-primed PCR in a single tube reaction. The amplified DNA was used as a complex probe mixture for fluorescent *in situ* hybridization on first meiotic metaphase spreads of lines carrying 5BL as a distinctive marker. Hybridization signals were observed, specifically, along the entire 5BL. In some of the cells, labeling was also detected in two bivalents, presumably those of the 5B "homoeologues" (partial homologues) found in common wheat (5A and 5D). The probe also revealed discrete domains in tapetal nuclei at interphase, further supporting the probe's high specificity. These data suggest that chromosome- and homoeologous group-specific sequences are more abundant in 5BL than genome-specific sequences. Chromosome-painting probes, such as the one described here for 5BL, can facilitate the study of chromosome evolution in polyploid wheat.

During the estimated 130–230 million years of angiosperm evolution (1), plant genomes have evolved to sizes ranging from ≈0.1 pg (*C* value) to >100 pg (2). This size variation reflects massive changes in repetitive DNA content and changes in 2*n* chromosome number (from four to several hundreds) due to polyploidy or aneuploidy (3). In contrast to plant genomic plasticity, mammalian species, which appeared 200–250 million years ago (4), share genomes of similar size (1.5–6.0 pg) (5) and are exclusively diploid. Gene order along the chromosomes (synteny) is highly conserved across mammalian orders (6, 7), whereas in angiosperms synteny is not conserved beyond the family level (8). Within the Gramineae (Poaceae), species from different botanical tribes such as the Triticeae (wheat, rye, and barley), the Maydeae (maize), the Andropogoneae (sorghum), and the Oryzeae (rice) have maintained synteny across large chromosomal regions (9–11), despite having different chromosome numbers and genome sizes that range from 0.60 pg (1*C*) in rice to 17.30 pg in wheat (2). The emerging view of genome evolution in higher plants is that speciation correlates with rapid changes in repetitive DNA, whereas the linear order of the genes and the genes themselves are slower to evolve (12). Therefore, the characterization of genome- or chromosome-specific sequences, the determination of their chromosomal location and amplification mechanisms are essential to our understanding of plant genome evolution. Because of the repetitive nature of these sequences, neither classical linkage

mapping analysis nor chromosome walking is suitable for studying karyotype evolution. *In situ* hybridization (ISH), on the other hand, has become the method of choice to identify and map directly repetitive as well as low-copy and single-copy sequences (13, 14). Using ISH in cereals, repetitive DNA that is organized in simple tandem arrays was correlated mainly with paracentromeric and telomeric heterochromatic regions (15–17), whereas another class of repetitive DNA hybridized uniformly along the chromosome arms (17–20). The repetitive probes used in these studies usually map over most, if not all, of the chromosome complement and also cross-hybridize to related genomes. Labeling of defined chromosomal regions has been obtained by using probes from highly repetitive DNA sequences, such as rRNA-encoding DNA (21), or from low-copy sequences, such as the genes encoding for secalins (22) or hordeins (23). Painting of an entire specific chromosome within its own complement, a tool that has been developed and used in mammals very successfully (24–27), should greatly contribute to the understanding of the unique aspects of plant karyotype evolution. To date, the genomic probing method (28) has allowed the identification of alien chromosomes, or chromosome segments, in a host genome by using total genomic DNA from the alien species as a probe. However, to our knowledge, no chromosome-painting probe that labels a specific chromosome, throughout its entire length, within a given species has been developed in plants.

We report here the successful painting of a chromosome arm—namely, the long arm of chromosome 5 of the wheat B genome (5BL) by combining the following approaches: (*i*) microdissection of 5BL isochromosomes, carrying two homologous 5BL arms; (*ii*) general DNA amplification of the dissected chromosomes using a degenerate oligonucleotide-primed PCR (DOP-PCR); and (*iii*) fluorescent *in situ* detection of 5BL with the amplified DNA as a complex probe.

MATERIALS AND METHODS

Genetic Stocks. Common wheat, *Triticum aestivum* L., is an allohexaploid (2*n* = 6*x* = 42, genome AABBDD) that has evolved through the hybridization of three closely related diploid species (29). The capability of particular chromosomes of the A, B, or D genomes to compensate for the absences of other particular chromosomes in the remaining two genomes has facilitated the development of a wide array of viable aneuploid stocks (30): nullisomics, monosomics, trisomics, tetrasomics, as well as lines carrying isochromosomes (chromosomes with homologous arms) and telochromosomes (chromosomes with a single arm). We have made

Abbreviations: DOP-PCR, degenerate oligonucleotide-primed PCR; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; ISH, *in situ* hybridization; 5BL, long arm of chromosome 5 of the wheat B genome.

use of the distinctive meiotic behavior of isochromosomes and telochromosomes at metaphase I, where they lie at the periphery of the equatorial plate, to identify 5BL. Hence, the following three lines of the Chinese Spring wheat cultivar were used for microdissection and ISH detection: monoisosomic 5BL, carrying one 5BL isochromosome; diisosomic 5BL, having two 5BL isochromosomes; and monotelosomic 5BL, carrying one 5BL telochromosome.

Chromosome Microdissection. Anthers were removed from fresh spikes of 5BL monoisosomic line of Chinese Spring and fixed in an ethanol/acetic acid, 3:1, solution for 1 min. Cell spreads were prepared in 20% (vol/vol) acetic acid between a coverslip sandwich as described (31). The upper coverslip was removed after immersion in liquid nitrogen for 2 min. Squashes were dehydrated in a series of 70% (vol/vol) and 90% (vol/vol) ethanol, 10 min each, and then kept in 100% ethanol at −80°C. Microdissection was done on an inverted microscope (Zeiss Axiovert 35; ×400) with microneedles controlled by a micromanipulator (Eppendorf 5170). The needles were prepared by extending borosilicate glass rods with a micropipette puller to form a tip of 0.5–1.5 μm. Before use, microneedles were UV-treated for 1 hr (Stratalinker, Stratagene). The tip of the needles carrying the dissected chromosome was broken off into a 0.5-ml siliconized microcentrifuge tube containing 20 μl of a proteinase-K solution at 50 μg/ml (Merck). In a typical microdissection session up to 10 chromosomes are pooled into a tube.

DOP-PCR Amplification. The collection drop with the microdissected chromosomes was incubated at 50°C for 1 hr, and then the proteinase-K was heat-inactivated at 90°C for 10 min. DOP-PCR was done as described (32) with minor modifications. Briefly, a first round of PCR amplification was performed in the same tube by adding 1.5 μM degenerate primer (5'-CCGACTCGAGNNNNNNATGTGG-3'), 200 μM of each dNTP, 2 mM $MgCl_2$, 5 μl of 10× *Taq* buffer (Promega), 2.5 units of *Taq* DNA polymerase (Promega), and H_2O to a final volume of 50 μl. The sample was overlaid with mineral oil and heated for 3 min at 90°C followed by 5 cycles of 1 min at 94°C, 1.5 min at 30°C, 3-min transition at 30–72°C, and 3 min at 72°C, followed by 25 cycles of 1 min at 94°C, 1 min at 62°C, and 2.5 min at 72°C with a final extension of 10 min at 72°C. The DOP-PCR technique allows priming at multiple sites along the template by placing a random sequence adjacent to a short specified sequence in the 3' primer end. The annealing of the 3'-specific hexamer was stabilized by the annealing of the adjacent random hexamer at the initial low temperature cycles (30°C). To increase the amount of product, 2 μl of the first PCR amplification mixture was subjected to a 20-cycle second-round PCR under the same conditions of high-stringency cycles of the first round. Because of the general amplification capability of the DOP-PCR, special precautions were taken to eliminate DNA from exogenous sources. Microdissection, solutions, and PCR assays were set up in laminar flow hood. Slides and coverslips were baked at 180°C for 3 hr, and plastic items were autoclaved. To test the level of DNA contamination, we included in each experiment a blank reaction with no added DNA and determined the PCR products by ethidium bromide staining of an agarose gel. The rate of false-positive results was drastically reduced by treating the equipment with a combination of 1 M HCl and UV light.

Fluorescence ISH. About 100 ng of DNA from the second-round PCR products were purified through a Promega Wizard PCR preps column and then labeled by the random-hexamer method (33) with 4-rhodamine-dUTP (Amersham). The labeled probe DNA was precipitated in the presence of 2 μg of calf thymus DNA to remove unincorporated nucleotides, washed in 70% ethanol, dried, and resuspended in water. Meiotic chromosome spreads were prepared and pretreated as described (34), and the chromosomal DNA was denatured

in 70% (vol/vol) formamide in 2× standard saline/citrate (SSC) at 68°C for 3.5 min and dehydrated in an ethanol series. Slides were then preheated in a humid chamber to the hybridization temperature (42°C). The labeled probe DNA was denatured by boiling for 7 min, quenched on ice for 5 min, and made up into 2× SSC. The ice-cold hybridization mix was then added to the preheated slides, coverslips were applied, and hybridization was done in a humid chamber floating in a water bath at 42°C overnight. Slides were then washed twice in 2× SSC at 65°C for 15 min, followed by 5 min in 4× SSC/0.2% Tween-20 at room temperature. The spreads were then stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 2 μg/ml) for 15 min and mounted in Vecta-shield (Vector Laboratories).

RESULTS

Microdissection. The pairing of the two homologous chromosome arms of an isochromosome leads via chiasma formation to the production of a ring univalent, easily identified in meiotic preparations by its position at the periphery of the equatorial plate (Fig. 1 *a–c*). Hence, the identity of the microdissected 5BL isochromosome was confirmed without the use of banding techniques that may depurinate the DNA. In the results presented below six isochromosomes were used in a single tube reaction.

DOP-PCR Amplification. DOP-PCR amplification of the dissected isochromosomes resulted in continuous size fragments ranging from 150 to 700 bp, the majority of the products being ≈400 bp long (data not shown). A similar pattern of amplification was reported for the same primer on microdissected human chromosomes (25) and on flow-sorted plant chromosomes (35, 36), indicating that this technique overcomes the level of species-dependent genome complexity and allows uniform amplification along the template DNA. Southern blots of the amplified DNA gave a positive signal when probed with wheat genomic DNA, whereas no signal was seen in identically processed reactions where dissected chromosomes were not added (data not shown).

Fluorescence ISH. Products of the second-round PCR were labeled with rhodamine and used as a complex probe mixture for ISH onto meiotic chromosome spreads of wheat lines carrying either one or two 5BL isochromosomes or one 5BL telochromosome. Cells at first meiotic metaphase stained with the DNA-specific dye DAPI showed blue fluorescence homogeneously distributed along the chromosomes; no differences were detected between the bivalents and the 5BL ring univalent(s) or the 5BL telocentric univalent. When the same meiocytes were visualized by fluorescence to detect the rhodamine labeling, a bright red signal was observed specifically along the entire length of the 5BL iso- and telochromosomes (Fig. 1 *d* and *e*). The rest of the chromosomes were not labeled except for two bivalents that were detected in some of the cells (Fig. 1*e*), most probably the 5B "homoeologues" (partial homologues) on the A and D genomes. In meiocytes of the 5BL diisosomic line where the two isochromosomes formed a bivalent, three bivalents were highlighted (Fig. 1*f*). In some cells, the paracentromeric and telomeric regions of most chromosomes were labeled (data not shown). The hybridization signal was also observed at interphase of the binucleated tapetal cells surrounding the meiocytes. In these cells, although DAPI staining did not reveal differential chromatin condensation within the nuclei, discrete domains of hybridization were disclosed by rhodamine fluorescence (Fig. 1*g*).

DISCUSSION

We have reported here on the amplification of DNA from a specific wheat chromosomal arm and its use as a probe for
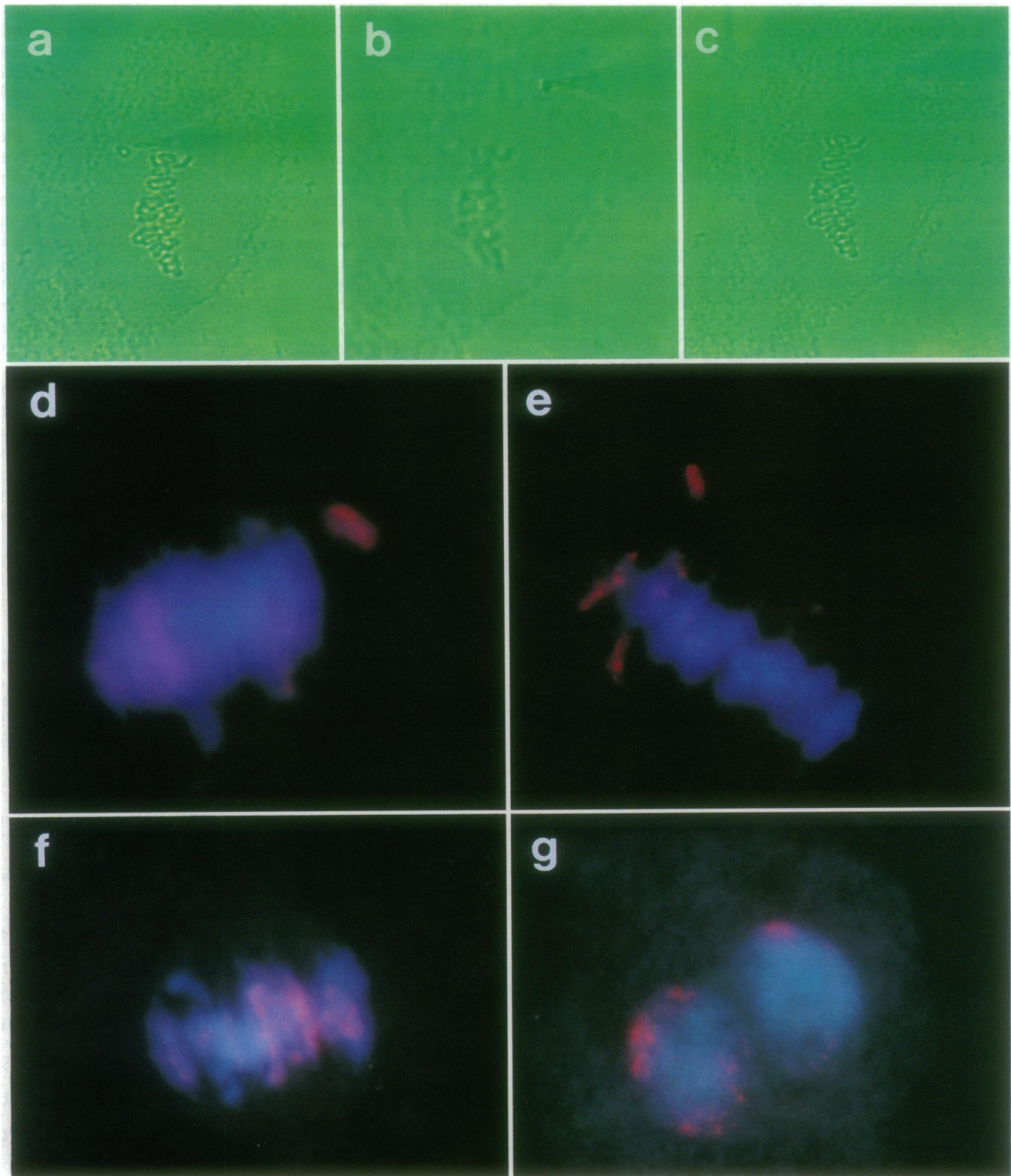
FIG. 1. First meiotic metaphase (*a–f*) and tapetal cells (*g*) of monoisosomic, diisosomic, and monotelosomic wheat lines for 5BL. (*a–c*) Sequential photographs illustrating the microdissection of the 5BL isochromosome. (*d–g*) Double-exposure photographs visualizing both the DAPI (blue) and rhodamine (red) fluorescence. (*d*) Monoisosomic 5BL showing the isochromosome separated from the metaphase plate. (*e*) Monotelosomic 5BL with the telochromosome at the periphery of the equatorial plate. (*f*) Diisosomic 5BL with the two isochromosomes forming a single bivalent. (*g*) Interphase nuclei in a tapetal cell of monoisosomic 5BL line.

chromosome painting. Previous works on microdissection of plant chromosomes have used microcloning (37) or a ligation-mediated PCR approach to amplify the dissected material (38–40). These works did not provide strong evidence for the enrichment of clones specific to the microdissected chromosomes. Both microcloning and ligation-mediated PCR in-

volve restriction and ligation of micro amounts of DNA, and different combinations of restriction enzymes and adaptors have to be used to obtain a genomic library that covers a whole chromosome. The multiplicity of handling steps involving the microdissected DNA increases the likelihood of contamination, beside being time-consuming. In contrast,

DOP-PCR is relatively simple and does not involve restriction or ligation steps. Although preferential amplification of some sequences cannot be ruled out, the obtained pattern of ISH throughout 5BL provides direct evidence for successful amplification along the whole microdissected DNA template. Flow-sorted chromosomes have also been used to generate chromosome-enriched libraries in plants (35, 41). However, one limitation of flow sorting is that chromosomes with similar DNA content are poorly resolved. The ISH data shown here provide a good quantitative assay to test the actual level of chromosome-specificity in the dissected DNA. This specificity was further demonstrated by the fact that no unlabeled (block) wheat DNA had to be included in the hybridization mixture to improve the contrast between the target chromosome(s) and the rest of the genome. Cloning of such DOP-PCR products may allow the production of a chromosome-arm-enriched library. This could facilitate high-resolution mapping, positional cloning of genes in a given region, and marker-assisted selection in breeding programs. In the case of 5BL, several target genes are available, such as the most potent chromosome-pairing regulator *Ph1* (42), as well as several other major genes and quantitative trait loci for agronomically important characters (43).

The differential labeling of the 5BL iso- and telochromosomes (Fig. 1 *d* and *e*) was not caused by their separation from the equatorial plate and, hence, a greater accessibility to the probe. No such labeling was found in nonrelated chromosomes that occasionally (or at anaphase) were apart from the bulk of the chromosome complement (data not shown). Moreover, when two 5BL isochromosomes paired in a bivalent located at the metaphase plate, rhodamine labeling was still restricted to three bivalents (Fig. 1*f*), presumably those involving the two 5BL isochromosomes and the two pairs of homoeologues (5A and 5B).

The occurrence of discrete labeled domains at interphase of the binucleated tapetal cells (Fig. 1*g*), despite homogeneous DAPI staining, is additional evidence for the probe specificity. This finding also suggests that at interphase individual chromosomes tend to lie in restricted nuclear areas.

The pattern of ISH suggested that the PCR products included a mixture of sequences specific to 5BL and its homoeologues. This mixture contained also, though to a lesser extent, sequences common to the whole chromosome complement as deduced by the labeling, in some cells, of telomeric and paracentromeric regions of non 5BL-related chromosomes. These nonspecific sequences are presumably highly repetitive DNA families.

Two bivalents were labeled in addition to 5BL (Fig. 1 *e* and *f*). It is most likely that these bivalents were 5A and 5D, which share extensive sequence homology with 5BL (44–46). However, the labeling of the homoeologous chromosomes appeared somewhat weaker than that of 5BL, indicating the existence of chromosome-specific sequences in the probe. This differential painting intensity needs to be further investigated in the appropriate genetic stocks where the 5BL homoeologues can be easily identified.

The nature of the chromosome- and group-specific sequences is unknown. Such sequences are obviously present in our probe, and their nature should be elucidated by cloning, sequencing, and mapping of individual PCR products. We do not expect that these sequences are of the retroelement type, a major component of plant repetitive DNA (47) because, after transposition, such sequences are expected to be spread throughout the whole genome. The fact that 5B was the only labeled chromosome of the B genome indicates that chromosome-, or group-specific sequences are more abundant in this chromosome arm than genome-specific sequences. We presume that these chromosome-, or group-specific sequences consist of families of repeated sequences

locally amplified along the chromosomes (48). The timing of amplification of these sequences relative to the speciation events that gave rise to the A, B, and D genomes would determine whether those became group- or chromosome-specific sequences. Although, in the Triticeae, group-specific sequences were produced in the common ancestral diploid species, chromosome-specific ones may have evolved after speciation and continued to change at the polyploid level. The continued amplification at the polyploid level is evident by the higher DNA content of the B genome relative to the A and D genomes and to that of *Aegilops speltoides* (49), the putative diploid donor of the B genome. If chromosome-specific sequences play an important role in meiotic pairing, then further chromosome-specific modifications at the polyploid level may have been advantageous in facilitating the action of genes, such as *Ph1*, which, at meiosis, suppresses the pairing of homoeologous chromosomes and thereby restricts it to homologues.

The chromosome-painting probe we developed has a great potential to study interphase nuclear architecture, as well as to identify chromosomal rearrangements, interstitial deletions, and translocation breakpoints that are unidentifiable by standard chromosome-banding analysis. Moreover, this probe can be used to map karyotypic changes during the evolution of the Gramineae and to estimate the conservation of linkage blocks between distantly related species. It also may facilitate the identification, exact chromosomal allocation, and size estimation of small alien chromosome segments that were introgressed into the genome of cultivated wheat in breeding programs.

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