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Flow Sorting of Mitotic Chromosomes in Common Wheat (*Triticum aestivum* L.)

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ABSTRACT

The aim of this study was to develop an improved procedure for preparation of chromosome suspensions, and to evaluate the potential of flow cytometry for chromosome sorting in wheat. Suspensions of intact chromosomes were prepared by mechanical homogenization of synchronized root tips after mild fixation with formaldehyde. Histograms of relative fluorescence intensity (flow karyotypes) obtained after the analysis of DAPI-stained chromosomes were characterized and the chromosome content of all peaks on wheat flow karyotype was determined for the first time. Only chromosome 3B could be discriminated on flow karyotypes of wheat lines with standard karyotype. Remaining chromosomes formed three composite peaks and could be sorted only as groups. Chromosome 3B could be sorted at purity >95% as determined by microscopic evaluation of sorted fractions that were labeled using C-PRINS with primers for GAA microsatellites and for *Afa* repeats, respectively. Chromosome 5BL/7BL could be sorted in two wheat cultivars at similar purity, indicating a potential of various wheat stocks for sorting of other chromosome types. PCR with chromosome-specific primers confirmed the identity of sorted fractions and suitability of flow-sorted chromosomes for physical mapping and for construction of small-insert DNA libraries. Sorted chromosomes were also found suitable for the preparation of high-molecular-weight DNA. On the basis of these results, it seems realistic to propose construction of large-insert chromosome-specific DNA libraries in wheat. The availability of such libraries would greatly simplify the analysis of the complex wheat genome.

GENE mapping and isolation in wheat (*Triticum aestivum* L. Em. Thell., $2n = 6x = 42$) is hampered by the complex genome. The complexity is due to both the allohexaploid nature, *i.e.*, the presence of three homeologous genomes, A, B, and D, and the enormous size of the genome (1C $\sim 16 \times 10^9$ bp, Bennett and Smith 1976). Until now, several genetic linkage maps of wheat have been published (Devos and Gale 1993; Gill *et al.* 1996; Cadalen *et al.* 1997; Röder *et al.* 1998). However, their density needs to be increased to permit effective marker-assisted breeding as well as map-based cloning of genes of interest. In wheat, Röder *et al.* (1998) have found nonhomogenous distribution of microsatellite markers among individual chromosomes, which may complicate saturation of maps in certain areas of genome.

Fractionation into individual chromosomes is an attractive route to simplify the analysis of the complex wheat genome and to facilitate targeted isolation of molecular markers. Although this can be done by microdissection (Stein *et al.* 1998), flow cytometric sorting appears to be more attractive as large numbers of chromosomes can be purified in a short time (Doležel *et al.* 1999). Flow cytometric analysis and sorting of mitotic chromosomes (flow cytogenetics) were originally developed for humans (Carrano *et al.* 1979) and subsequently modified for farm animals (Dixon *et al.* 1992; Burkin *et al.* 1997). Flow cytometric classification of chromosomes (flow karyotyping) has proven to be a useful tool for detection of numerical and structural chromosome aberrations (Boschman *et al.* 1992). Flow-sorted chromosomes were used for gene mapping (Lebo 1982), construction of chromosome-specific DNA libraries (Van Dilla and Deaven 1990), including large-insert (YAC) DNA libraries (McCormick *et al.* 1993), generation of chromosome painting probes (Ferguson-Smith 1997), and targeted generation of molecular markers (Lan *et al.* 1999).

After the first report of De Laat and Blaas (1984), flow cytogenetics has been developed for 12 plant species (Doležel *et al.* 2000), which include economically important crops such as tomato (Arumuganathan *et al.* 1994), maize (Lee *et al.* 1996), and barley (Lysák *et al.* 1999). Sorted plant chromosomes were used for physical mapping (Macas *et al.* 1993), isolation of chromosome-specific probes (Arumuganathan *et al.* 1994), and for construction of chromosome-specific DNA libraries (Macas *et al.* 1996).

In wheat, two groups (Wang *et al.* 1992; Lee *et al.* 1997; Schwarzacher *et al.* 1997; Gill *et al.* 1999) reported flow cytometric analysis of chromosomes. Nevertheless, until now the flow karyotype (a distribution of relative chromosome DNA content obtained by flow

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cytometric analysis) of wheat has not been described accurately. Thus it was not clear which chromosomes and/or chromosome groups were sorted. In this work, we have developed a novel procedure for preparation of chromosome suspensions in wheat and have analyzed isolated chromosomes using flow cytometry. Chromosomes were sorted from individual peaks observed on high-resolution flow karyotypes and were identified using PRINS (primed *in situ* labeling) and PCR with specific primers. Wheat lines with standard karyotype as well as lines carrying chromosome translocations were analyzed to evaluate their potential for sorting of individual chromosomes.

MATERIALS AND METHODS

Plant material: Seeds of wheat (*T. aestivum* L., $2n = 6x = 42$) cv. Chinese Spring with a standard karyotype and cv. Cappelle Desprez, which carries a translocation chromosome 5BL/7BL, were kindly provided by Dr. R. Koebner (JIC, Norwich, UK). Seeds of wheat cultivars Saxana, Titus, and Jubilar were obtained from Dr. Z. Stehno (RICP, Prague, Czech Republic). Cv. Jubilar carries a translocation chromosome 5BL/7BL. All seeds were germinated in the dark at 25 ± 0.5° on moistened filter paper in glass petri dish for 2–3 days to achieve optimal root length (2–3 cm).

Cell cycle synchronization: All incubations were performed in the dark at 25 ± 0.5°, and all solutions were aerated. Seedlings were transferred to an open mesh basket positioned on a plastic tray filled with Hoagland's solution (Gamborg and Wetter 1975) containing hydroxyurea (HU) at various concentrations (1.0, 1.5, 2.0, 2.5, 3, 4, and 5 mm) and incubated for 18 hr. The roots were then washed in distilled water and immersed in HU-free Hoagland's solution. Samples of root tips were taken at 1-hr intervals up to 10 hr for analysis of cell cycle synchrony and mitotic activity. To accumulate cells at metaphase, seedlings were transferred 6, 6.5, 7, 7.5, or 8 hr after the recovery from HU block to Hoagland's solution containing 2.5 μm amiprophos-methyl (APM). This concentration was found to be optimal for metaphase arrest in preliminary experiments. Samples of root tips were taken 2, 3, or 4 hr after the incubation in APM to determine the frequency of metaphases. To improve chromosome spreading, the effect of overnight treatment in ice water (1–2°) after APM treatment was evaluated. To determine mitotic activity and frequency of metaphases, root tips were fixed in ethanol:acetic acid (3:1) and then squash preparations were prepared according to the Feulgen procedure. One thousand cells per slide and five preparations per variant were analyzed; each experiment was repeated three times.

Preparation of chromosome suspensions: Roots were cut 1 cm from the tip and fixed for periods from 10 to 45 min at 5° in various concentrations of formaldehyde (1, 2, 3, or 4%) in Tris buffer (10 mm Tris, 10 mm Na_2EDTA, 100 mm NaCl, 0.1% Triton X-100, pH 7.5). After three 5-min washes in Tris buffer, meristem tips (1 mm) of 30 roots were cut and transferred to a tube containing 1 ml of LB01 lysis buffer (Doležel et al. 1989) of the following composition: 15 mm Tris, 2 mm Na_2EDTA, 80 mm KCl, 20 mm NaCl, 0.5 mm spermine, 15 mm β-mercaptoethanol, 0.1% Triton X-100, pH 9. The chromosomes were released by homogenization with a Polytron PT 1200 homogenizer (Kinematica AG, Littau, Switzerland) at 9500 rpm for 10 sec. The suspension was passed through 50-μm nylon mesh to remove large tissue and cellular fragments. For checking chromosome morphology and concentration, 10 μl of chromosome suspension was dried on microscope slides. After mounting in LB01 buffer containing 0.2 μg/ml DAPI (4′,6-diamidino-2-phenylindole), the slides were observed using a fluorescence microscope.

Flow cytometry: Chromosome analysis and sorting were performed on the FACS Vantage flow cytometer (Becton Dickinson, San José, CA) equipped with argon laser set to multiline UV and 300 mW output power. A solution of 40 mm KCl and 10 mm NaCl was used as a sheath fluid. The suspension of isolated chromosomes was stained with DAPI at a final concentration of 2 μg/ml and analyzed at rates of 200–400 particles/sec. DAPI fluorescence was measured through a 424/44 bandpass filter in front of fluorescence 1 (FL1) detector. Relative fluorescence intensities, which corresponded to relative DNA content of gated populations, were acquired on histograms of FL1 pulse area (FL1-A). Approximately 20,000–50,000 chromosomes were analyzed in each sample. For chromosome sorting, gates were set on a dot plot of FL1-A *vs.* FL1 pulse width (FL1-W).

Cycling PRINS: Two thousand chromosomes were sorted on a microscope slide into a 15-μl drop of PRINS buffer supplemented with 5% sucrose (Kubaláková et al. 1997). After air drying, a Frame-Seal incubation chamber (MJ Research, Watertown, MA) was stuck to the slide over the dried drop containing sorted chromosomes, and 25 μl of PRINS reaction mix was placed into the frame. The reaction mix consisted of 100 μm dCTP, dGTP, 2 μm fluorescein-12-dUTP, fluorescein-12-dATP, 34 μm dTTP, dATP, 2 μm $(GAA)_7$ and $(CTT)_7$ primers, or AS1 and AS2 primers (Nagaki et al. 1998), 1.5 U/25 μl Dynazyme II DNA polymerase (Finnzymes OY, Finland), and 4 mm $MgCl_2$ in 1× PCR buffer. The reaction was performed on PTC 100 cycler (MJ Research) equipped with an actively controlled heated plate. The first reaction cycle consisted of 5 min at 94°, 5 min at 55°, and 10 min at 72°, followed by 1 min at 91°, 1 min at 55°, and 3 min at 72° during eight cycles. In the final cycle, the annealing was prolonged to 5 min and extension was prolonged to 10 min. Reaction with microsatellite primers gave stronger and sharper signals when the duration of each step (denaturation, annealing, and extension) was shortened to 45 sec and the number of cycles increased to 30. The reaction was stopped in a stop buffer (0.5 m NaCl, 0.05 m Na_2EDTA, pH 8.0) for 5 min at 70°, and the slides were washed in a wash buffer (0.1 m maleic acid, 0.15 m NaCl, 0.05% Tween-20, pH 7.5) at room temperature for 5 min. Slides were counterstained with propidium iodide (PI) at 0.2 μg/ml and mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, CA).

Fluorescence microscopy: The preparations were evaluated using Olympus BX 60 microscope equipped with filter sets appropriate for fluorescein and PI. The images of fluorescein and PI fluorescence were acquired separately with a black and white CCD camera, which was interfaced to a PC running ISIS software (Metasystems, Altlussheim, Germany). The images were superimposed after contrast and background optimization.

Polymerase chain reaction: The number of chromosome types represented by each peak on flow karyotype was estimated according to its area. Amounts of chromosomes corresponding to 1000 chromosomes of each type (*i.e.*, 4000 for peak I, 6000, 10,000, and 1000 for peaks II, III, and IV, respectively) were sorted into 0.5-ml PCR tubes containing 40 μl of sterile deionized water. The tubes with sorted chromosomes were frozen and kept at −20°. Before reaction, the chromosomes were thawed and PCR premix was added to reach the reaction volume of 100 μl. The final concentrations of the reagents were as follows: 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mm dNTPs, 5 units/

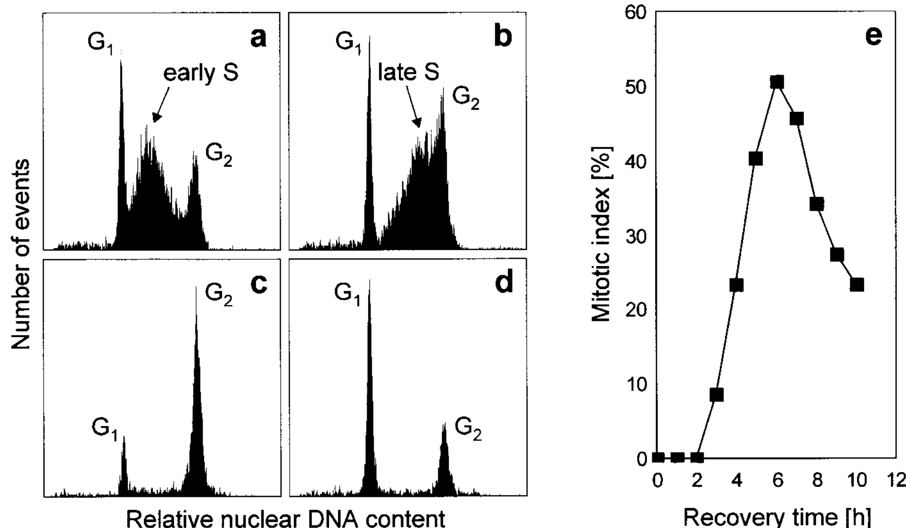

Figure 1.—Cell cycle (a–d) and mitotic (e) synchrony observed in root tip meristems of wheat after hydroxyurea treatment (2.5 mm/18 hr). (a–d) Histograms of relative DNA content were obtained after flow-cytometric analysis of nuclei isolated from root tips. (a) Three subpopulations of nuclei could be detected at the time of release from the block: G_1, early S, and G_2. The cells at early S represented the synchronized subpopulation; (b–d) samples taken during a recovery from the hydroxyurea block: (b) 2 hr; (c) 4 hr; (d) 8 hr. Most of root tips cells synchronously transited S, G_2, and M phases. (e) Mitotic activity was determined on Feulgen-stained squash preparations made from root tips sampled at 1-hr intervals after the removal.

100 μl *Taq* DNA polymerase (Sigma, St. Louis). Primers for chromosome-specific microsatellite markers (Röder *et al.* 1998) were used at 0.5 μm. PCR was performed under the following conditions: denaturation step 3 min at 94° followed by 35 cycles consisting of 1 min denaturation at 92°, 1 min annealing at 52, 55, or 57°, 2 min extension at 72°, and final extension at 72° for 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Isolation of high-molecular-weight DNA: Approximately 8×10^5 chromosomes were sorted into 640 μl of ice-cold 1.5× IB buffer (1× IB: 15 mm Tris, 10 mm EDTA, 130 mm KCl, 20 mm NaCl, 1 mm spermine, 1 mm spermidine, 15 mm β-mercaptoethanol, 0.1% Triton X-100, pH 9.4). After pelleting at 200 × *g* for 30 min at 4°, the chromosomes were resuspended in 40 μl of IB, warmed to 50°, and mixed with an equal amount of prewarmed 1.5% low-melting-point (LMP) agarose made in IB. The mixture was poured into an 80-μl plug mold to form an agarose plug. The plug was solidified on ice and incubated in 1 ml of lysis buffer (0.5 m EDTA, 1% lauroylsarcosine, 0.1 mg/ml proteinase K, pH 8–9) at 37° for 2 days. The quality of chromosomal DNA was checked using the CHEF-DR II pulsed-field gel electrophoresis system (Bio-Rad, Hercules, CA). To prove the purity of the high-molecular-weight (HMW) DNA, prepared DNA samples were digested with *Hin*dIII restriction endonuclease (MBI Fermentas, Vilnius, Lithuania). Agarose plugs were washed for 5 hr in five changes of ice-cold TE buffer (10 mm Tris, 1 mm EDTA, pH 8.0). The DNA in the plugs was digested with 5 units of enzyme for 5 min or with 25 units for 2 hr at 37°. Control reaction without addition of enzyme was included. Quality of the digested DNA was checked using pulsed-field gel electrophoresis (PFGE). The electrophoresis was run on a 1% agarose gel in 0.5× TBE, at 13.5°, 6 V/cm with a 90-sec switch time for 20 hr.

RESULTS

Cell cycle synchronization and accumulation of root tip cells in metaphase: Flow cytometric analysis of isolated nuclei showed that root tip cells were traversing early S phase at the time of HU removal (Figure 1a), indicating that the cells did not remain arrested at the G_1/S interface until the end of HU treatment. After the removal of HU, the cells continued in synchronized progression through middle and late S phase, with most of the cells entering G_2 phase ~5 hr after the HU removal. Synchronized cells subsequently passed mitosis and entered the next G_1 phase (Figure 1, b–d). The highest degree of mitotic synchrony was obtained after the treatment with 2.5 mm HU for 18 hr, which resulted in a sharp peak of mitotic activity 7 hr after the removal from HU. At this point, >50% cells were found in mitosis (Figure 1e). The frequency of cells blocked with 2.5 μm APM in metaphase depended both on the timing and the length of the treatment. Prolongation of the APM treatment up to 4 hr resulted in increased frequency of metaphases (≤65%). However, it also resulted in increased occurrence of cells with single chromatids. The highest frequency of metaphases (50%) with a negligible occurrence of single chromatids was observed after a 2-hr treatment with 2.5 μm APM applied 4.5 hr after the removal from HU. Incubation of APM-treated roots in ice water improved chromosome spreading without a negative effect on the frequency of metaphase cells.

Preparation of chromosome suspensions: The extent of fixation with formaldehyde critically determined the quantity and the morphology of isolated chromosomes. Chromosome yield was higher after fixation with 1–2% formaldehyde for 10–15 min. However, isolated chromosomes were damaged and chromosome suspensions contained large amounts of chromosomal and nuclear debris. On the other hand, suspensions obtained after homogenization of root tips fixed with 2–4% formaldehyde for 30–45 min contained an increased number of chromosome clumps and intact cells. Fixation in 2% (v/v) formaldehyde for 20 min was selected as optimal producing $>5 \times 10^5$ chromosomes with well-preserved morphology from 30 root tips.

Flow karyotyping and chromosome sorting: High-resolution flow karyotypes were routinely obtained after the analysis of chromosomes isolated from all genotypes of wheat used in this study. Flow karyotype of cv. Chinese

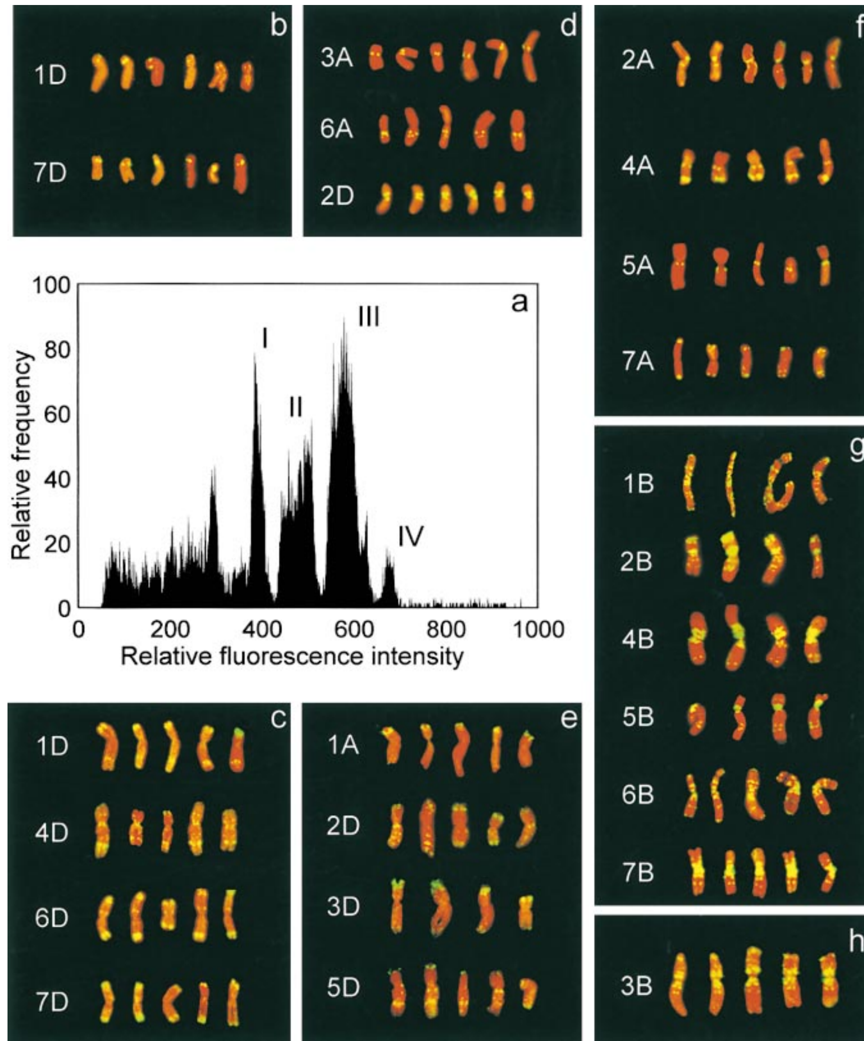

FIGURE 2.—Flow karyotype of *T. aestivum* cv. Chinese Spring (a) and images of chromosomes sorted from individual peaks resolved on the karyotype. Sorted chromosomes were identified after fluorescent labeling using C-PRINS with primers for GAA microsatellites (b, d, and f–h) or with primers for *Afa* repeats (c and e). The chromosomes were counterstained with propidium iodide. (a) Flow karyotype obtained after analysis of DAPI-stained chromosomes contains three dominant composite peaks (I, II, and III) representing various numbers of chromosome types and well-resolved peak IV, which represents chromosome 3B. (b and c) Chromosomes sorted from peak I; (d and e) chromosomes sorted from peak II; (f and g) chromosomes sorted from peak III; (h) Chromosomes 3B sorted from peak IV.

Spring consisted of three composite peaks (labeled I, II, and III) corresponding to various chromosome types and a peak IV corresponding to a single chromosome type (Figure 2a). In addition to chromosome peaks, composite peaks representing chromatids were visible. Chromosome content of individual peaks was determined after fluorescent labeling of flow-sorted chromosomes using cycling PRINS (C-PRINS) and after PCR on sorted chromosomes using primers for chromosome-specific markers. Chromosomes were sorted at a rate of ~5–10/sec. To achieve the highest count precision and purity, a "counter" sorting mode and one drop sort envelope were used for sorting.

C-PRINS with primers for GAA microsatellites on sorted chromosomes resulted in intensive and sharp signals on all of B genome chromosomes. With the exception of chromosome 1A, one to several bands were labeled on the A genome chromosomes. In addition, one or two bands were labeled on chromosomes 1D, 2D, and 7D (Figures 2, b, d, and f–h). GAA banding patterns were similar to those obtained after C banding (GILL *et al.* 1991) and after FISH with a probe containing a GAA satellite sequence (PEDERSEN and LANGRIDGE 1997). Chromosome 1A and some D chromosomes were not labeled by this procedure and were identified after C-PRINS with primers specific for repetitive *Afa* family sequences (Figure 2, c and e). Fluorescent banding patterns thus obtained were comparable to those observed after FISH with a probe containing *Afa* repeats (PEDERSEN and LANGRIDGE 1997). Combined use of C-PRINS with primers for GAA microsatellites and *Afa* repeats permitted unambiguous assignment of all wheat chromosomes to peaks on flow karyotype (Table 1). As can be seen, peak IV corresponded to chromosome 3B. The chromosome could be sorted at purity >95% as determined by C-PRINS.

In addition to PRINS, PCR with primers for chromosome-specific microsatellite markers was used to verify the assignment of chromosomes to the peaks on flow karyotype. Chromosome content of composite peaks as determined by C-PRINS was confirmed after PCR with chromosome-specific primers as shown in Figure 3a. Furthermore, the identity and purity of sorted chromosome 3B was confirmed (Figure 3b).

TABLE 1

Determination of chromosome content of the four peaks on flow karyotype of common wheat cv. Chinese Spring

	Genome/chromosome																				
	A							B							D						
Peak	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
I															X			X		X	X
II	X		X			X										X	X		X		
III		X		X	X		X	X	X		X	X	X	X							
IV										X											

Polymorphism of chromosome 3B: Flow karyotypes of cv. Chinese Spring and other wheat cultivars used in this study differed in the shape of composite peaks as well as in the position of peak IV corresponding to chromosome 3B (Figure 4, a and c). In case of cv. Cappelle Desprez, the chromosome 3B-representing peak could be discriminated only partially. This observation indicated that flow cytometry was sensitive enough to detect small differences in relative DNA content of wheat chromosomes. The polymorphism of chromosome 3B was confirmed after C-PRINS with primers for GAA microsatellites. Differences were observed in the number, position, and size of GAA-rich clusters (Figure 4d).

Flow karyotyping in translocation lines: The analysis of chromosomes isolated from cv. Cappelle Desprez and cv. Jubilar carrying translocation chromosome 5BL/7BL showed that in addition to chromosome 3B, the chromosome 5BL/7BL could also be discriminated and sorted (Figure 4, b and c). Microscopic analysis of sorted chromosome 5BL/7BL fractions after fluorescent labeling of GAA microsatellites using C-PRINS (Figure 4e) showed that the chromosome could be sorted at purity >91% in both cultivars.

Isolation of high-molecular-weight DNA: Wheat chromosomes were purified by flow sorting at rate of 50/sec. To increase sort yield, the "normal-r" sorting mode was used. Under these conditions, it was possible to sort 1.6×10^6 chromosomes during one working day. This quantity was sufficient to prepare two plugs for PFGE. The analysis by PFGE revealed that the majority of DNA did not migrate into the gel remaining in the sample well, which suggests the DNA to be of megabase size. To prove that the low migration ability was not due to an insufficient purity of the samples, the HMW DNA was digested by *Hin*dIII (Figure 5). After a 5-min incubation in *Hin*dIII buffer with 5 units of *Hin*dIII, the DNA was considerably digested whereas no digestion occurred in the control DNA incubated in buffer without addition of the enzyme. Nearly complete digestion was achieved after 2 hr of treatment with 25 units of *Hin*dIII. These results showed that the chromosome DNA was well accessible for the restriction endonuclease.

DISCUSSION

Two research groups previously reported preparation of chromosome suspensions in common wheat and their analysis by flow cytometry. In the first report, Wang *et al.* (1992) isolated mitotic chromosomes from suspension-cultured cells. The authors observed a number of

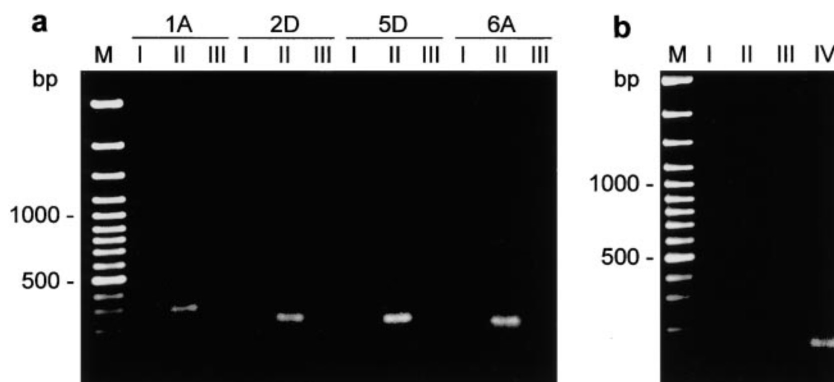

Figure 3.—Assignment of chromosomes 1A, 2D, 5D, and 6A to peaks on flow karyotype (a) and identification of sorted chromosome 3B by PCR (b). (a) The reaction was performed on 4000 sorted chromosomes from peak I (lanes I), 6000 sorted chromosomes from peak II (lanes II), and 10,000 sorted chromosomes from peak III (lanes III) with the following microsatellite primers: WMS136L, R, specific for chromosome 1A (lanes 1A); WMS349L, R, specific for chromosome 2D (lanes 2D); WMS190L, R, specific for chromosome 5D (lanes 5D); and WMS169L, R, specific for chromosome 6A (lanes 6A). PCR products were analyzed by agarose-gel electrophoresis. The results showed that chromosomes 1A, 6A, 2D, and 5D are comprised in peak II. (b) PCR reaction was performed on chromosomes sorted from peak I (lane I), peak II (lane II), peak III (lane III), and peak IV (lane IV) with microsatellite primers WMS 340L, R (Röder *et al.* 1998), which are specific for chromosome 3B. PCR products were analyzed by agarose gel electrophoresis. A band of 132 bp was obtained only with chromosomes sorted from peak IV.

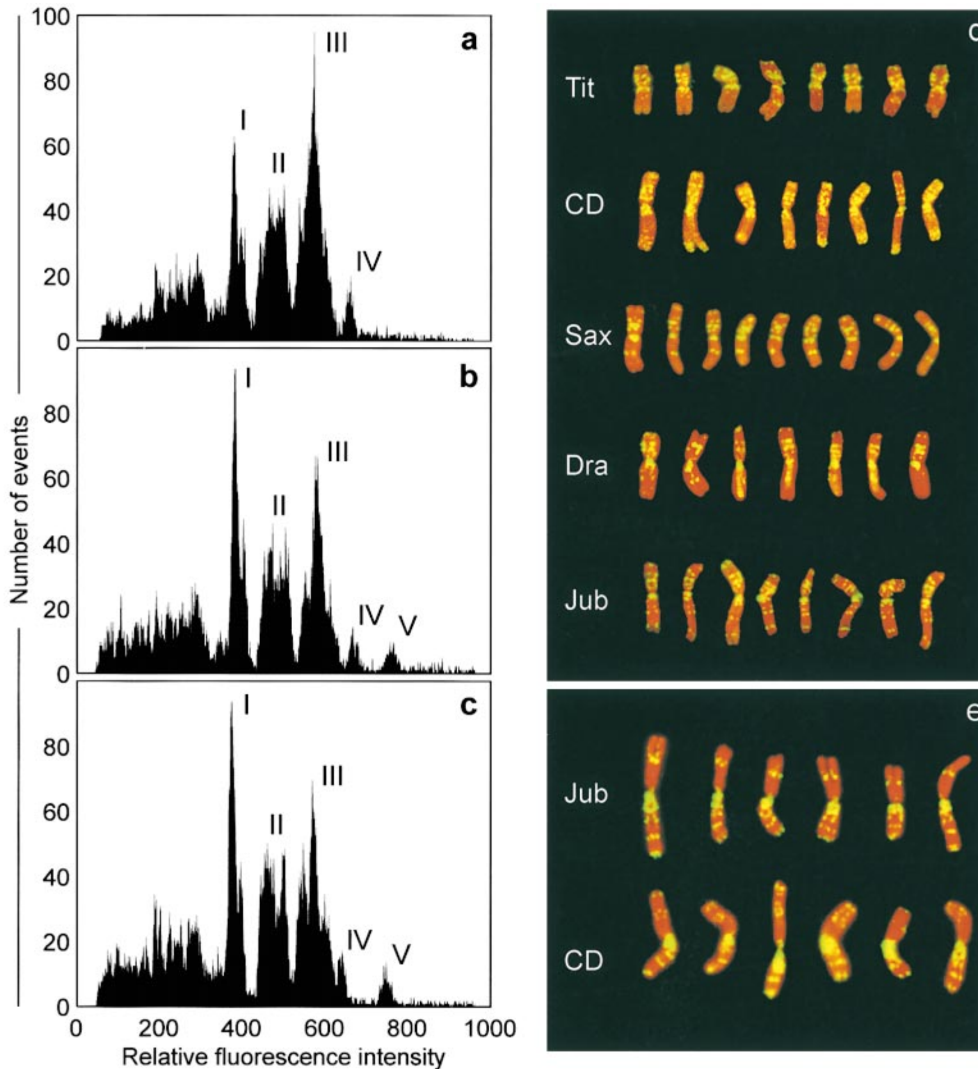

Figure 4.—Flow karyotypes obtained after analysis of DAPI-stained chromosomes in three cultivars of wheat (a–c), and images of sorted chromosomes obtained after fluorescent labeling using C-PRINS with primers for GAA microsatellites (d and e). The chromosomes were counterstained with propidium iodide. Differences in the relative position of peak IV, which corresponds to chromosome 3B, can be seen on flow karyotypes of cv. Saxana (a), cv. Jubilar (b), and cv. Cappelle Desprez (c). Peaks representing chromosomes 5BL/7BL can be seen on flow karyotypes of cv. Jubilar (b) and cv. Cappelle Desprez (c). Images of chromosomes 3B sorted from five cultivars of wheat (d). Note the differences in the size and position of GAA clusters. (e) Images of chromosomes 5BL/7BL sorted from two wheat cultivars. Tit, Titus; CD, Cappelle Desprez; Sax, Saxana; Dra, Drake; Jub, Jubilar.

peaks on flow karyotype obtained after analysis of chromosome suspensions. However, they were not able to assign chromosome peaks to individual chromosomes (Schwarzacher *et al.* 1997). An alternative procedure for preparation of wheat chromosome suspensions from synchronized root tips was described by Lee *et al.* (1997). Although the flow karyotypes had relatively good resolution, the authors were only able to identify groups of peaks representing chromosomes and chromatids. However, they failed to determine the chromosome content of individual peaks. Neither research group identified the peak representing single chromosome 3B.

The high-yield procedure for preparation of wheat chromosome suspensions presented here is a modification of the method originally developed for *Vicia faba* (Doležel *et al.* 1992). Unlike in other protocols, intact chromosomes are mechanically released from synchronized root tips after a mild fixation with formaldehyde. A combined treatment with HU (DNA synthesis inhibitor) and APM (mitotic spindle inhibitor) results in effective cell cycle synchronization and accumulation of cycling cells in metaphase. The degree of mitotic synchrony in root tips achieved after HU treatment is comparable to other reports on cell cycle synchronization (Clain and Brulfert 1980; Pan *et al.* 1993). However, chromosome yield is about four times higher compared to the protocol developed by Gill *et al.* (1999).

Chromosome isolation from formaldehyde-fixed root tips offers many important advantages. Fixed chromosomes are resistant to mechanical shearing forces and thus can be released using a mechanical homogenizer. Isolated chromosomes are mechanically stable and can be stored and withstand shearing forces during flow sorting. This permits reanalysis of sorted fractions and even resorting, an approach that may be used to improve the purity in sorted fractions (Lucretti *et al.* 1993). It should also be noted that plant chromosomes isolated from formaldehyde-fixed root tips were shown to be suitable for scanning electron microscopy (Schubert *et al.* 1993), localization of DNA sequences using *in situ* hybridization (Fuchs *et al.* 1994) and PRINS (Kubaláková *et al.* 1997), and for immunolocalization

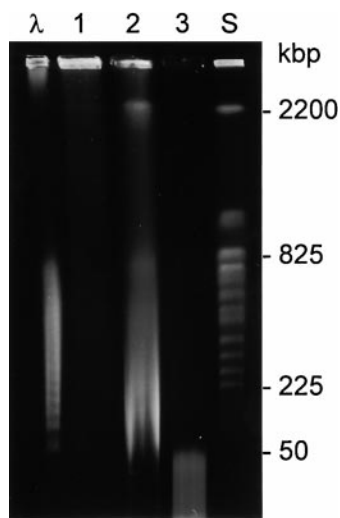

Figure 5.—Pulsed-field gel electrophoresis of high-molecular-weight DNA prepared from flow-sorted chromosomes after digestion by *Hin*dIII. Wheat HMW DNA in agarose plugs was incubated in *Hin*dIII buffer containing 0 (lane 1), 5 (lane 2), or 25 units (lane 3) of *Hin*dIII for 5 min (lanes 1 and 2) or 2 hr (lane 3) at 37°. The electrophoresis was run on a 1% agarose gel in 0.5× TBE at 13.5°, 6 V/cm with a 90-sec switch time for 20 hr. Lane λ, lambda ladder; lane S, chromosomes of *Saccharomyces cerevisiae* used as size markers.

of chromosomal proteins (Binarová *et al.* 1998). Our results obtained in five different genotypes of wheat proved that the procedure is not genotype dependent.

In this study, flow karyotype of wheat was described accurately and chromosome content of individual peaks was determined for the first time. The results indicated that in wheat with a standard karyotype only chromosome 3B could be sorted at high purity. Other chromosomes formed three composite peaks and could be sorted only as groups. Interestingly, peak I corresponded to four D-genome chromosomes. This gives an opportunity to separate part of the wheat D genome. Clear discrimination of chromosome 3B is possible due to its size and, hence, relative DNA content. According to Gill (1987), chromosome 3B is the longest chromosome in the karyotype of common wheat.

Although a large variation in the degree of chromatin condensation of sorted chromosomes was observed, it was possible to identify them using PCR and C-PRINS. The latter method facilitated quantitative determination of purity in sorted fractions. Because C-PRINS with primers for GAA repeats did not permit identification of all wheat chromosomes, we also used primers specific for repetitive *Afa* family sequences, first cloned from *Aegilops squarrosa* (Rayburn and Gill 1986). As shown previously by Pedersen and Langridge (1997), FISH with probes for GAA and *Afa* repeats is suitable for unambiguous identification of wheat chromosomes. In this study, C-PRINS was used for the first time to identify all wheat chromosomes. Compared to FISH (Pedersen and Langridge 1997), C-PRINS resulted in sharper GAA and *Afa* fluorescent banding patterns. A further advantage of C-PRINS over FISH is that it does not require labeled probes and is much faster.

A possibility to sort only a single chromosome type could compromise the usefulness of flow cytogenetics for gene mapping and isolation in wheat. However, this problem could be solved using various chromosome stocks, such as translocations and deletions. This approach was first proposed by Lucretti *et al.* (1993) and was applied successfully in *V. faba* (Doležel and Lucretti 1995), *Pisum sativum* (Neumann *et al.* 1998), and *Hordeum vulgare* (Lysák *et al.* 1999). An almost unlimited range of stocks is available in wheat (Sears 1954; Endo and Gill 1996), making this approach feasible. To develop a procedure for sorting of other chromosome types, we have analyzed chromosomes isolated from cv. Cappelle Desprez and cv. Jubilar. A peak corresponding to chromosome 5BL/7BL could be discriminated clearly and the chromosome could be sorted at high purity, thus extending the number of wheat chromosome types that can be sorted. Furthermore, Gill *et al.* (1999) reported sorting chromosome arm 1DS from a wheat ditelosomic line. The problem of sorting small chromosomes and/or chromosome arms is that they often fall into the region of chromatids, which compromises the purity in sorted fractions (our unpublished observations). On the other hand, large chromosomes, such as 3B and 5BL/7BL, can be sorted at very high purity. This approach could be feasible also for single chromosome arms, if lines carrying isochromosomes are used.

A rapid identification of chromosome 5BL/7BL showed that flow karyotyping might be an elegant tool to detect the presence of such a chromosome, or other translocation chromosomes that are larger than 3B, in wheat lines. It is also interesting to note that we have found flow karyotyping sensitive enough to detect polymorphism of chromosome 3B. The differences between chromosomes sorted from different wheat cultivars were observed also after GAA banding using C-PRINS with specific primers. The polymorphism in the number and position of diagnostic bands has been described already (Friebe and Gill 1994). However, this is the first report demonstrating suitability of flow cytometry to detect this type of variation.

The quality of DNA obtained from flow-sorted chromosomes is an important factor that determines the usefulness of flow cytogenetics in wheat. Our results demonstrate that chromosomes isolated and sorted according to our protocol are a suitable template for PCR. Thus flow-sorted chromosomes can be used for physical mapping of DNA sequences using PCR with specific primers and for construction of small insert DNA libraries after degenerate oligonucleotide-primed-PCR (Telenius *et al.* 1992). Furthermore, we have also shown that high-molecular-weight DNA could be prepared from flow-sorted wheat chromosomes and that the DNA

could be partially digested to obtain large fragments suitable for construction of large-insert DNA libraries.

In conclusion, a high-yield method for preparation of suspensions of intact wheat chromosomes, which are suitable for analysis and sorting using flow cytometry, has been developed. Wheat flow karyotype has been characterized and chromosome content of all peaks on the karyotype was determined for the first time. While only chromosome 3B could be sorted from wheat cultivars with a standard karyotype, the results indicate a possibility to sort other chromosome types using various chromosomal stocks. A possibility to prepare high-molecular-weight DNA from flow-sorted chromosomes opens a way for construction of large-insert chromosome-specific DNA libraries, which will aid in genome mapping and gene isolation in wheat.

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LITERATURE CITED

Arumuganathan, K., G. B. Martin, H. Telenius, S. D. Tanksley and E. D. Earle, 1994 Chromosome 2-specific DNA clones from flow-sorted chromosomes of tomato. Mol. Gen. Genet. **242:** 551–558.

Bennett, M. D., and J. B. Smith, 1976 Nuclear DNA amounts in angiosperms. Phil. Trans. Roy. Soc. Lond. Ser. B **274:** 227–274.

Binarová, P., B. Hause, J. Doležel and P. Dráber, 1998 Association of γ-tubulin with kinetochore/centromeric region of plant chromosomes. Plant J. **14:** 751–757.

Boschman, G. A., E. M. M. Manders, W. Rens, R. Slater and J. A. Aten, 1992 Semi-automated detection of aberrant chromosomes in bivariate flow karyotypes. Cytometry **13:** 469–477.

Burkin, D. J., P. C. M. O'Brien, T. E. Broad, D. F. Hill, C. A. Jones *et al.*, 1997 Isolation of chromosome-specific paints from high-resolution flow karyotypes of the sheep (*Ovis aries*). Chrom. Res. **5:** 102–108.

Cadalen, T., C. Boeuf, S. Bernard and M. Bernard, 1997 An intervarietal molecular marker map in *Triticum aestivum* L. Em. Thell. and comparison with a map from a wide cross. Theor. Appl. Genet. **94:** 367–377.

Carrano, A. V., J. W. Gray, R. G. Langlois, K. J. Burkhart-Schultz and M. A. Van Dilla, 1979 Measurement and purification of human chromosomes by flow cytometry and sorting. Proc. Natl. Acad. Sci. USA **76:** 1382–1384.

Clain, E., and A. Brulfert, 1980 Hydroxyurea-induced mitotic synchronization in *Allium sativum* root meristems. Planta **150:** 26–31.

De Laat, A. M. M., and J. Blaas, 1984 Flow-cytometric characterization and sorting of plant chromosomes. Theor. Appl. Genet. **67:** 463–467.

Devos, K. M., and M. D. Gale, 1993 Extended genetic maps of the homeologous group 3 chromosomes of wheat, rye and barley. Theor. Appl. Genet. **85:** 649–652.

Dixon, S. C., N. G. A. Miller, N. P. Carter and E. M. Tucker, 1992 Bivariate flow cytometry of farm animal chromosomes: a potential tool for gene mapping. Anim. Genet. **23:** 203–210.

Doležel, J., and S. Lucretti, 1995 High-resolution flow karyotyping and chromosome sorting in *Vicia faba* lines with standard and reconstructed karyotypes. Theor. Appl. Genet. **90:** 797–802.

Doležel, J., P. Binarová and S. Lucretti, 1989 Analysis of nuclear DNA content in plant cells by flow cytometry. Biol. Plant. **31:** 113–120.

Doležel, J., J. Číhalíková and S. Lucretti, 1992 A high-yield procedure for isolation of metaphase chromosomes from root tips of *Vicia faba* L. Planta **188:** 93–98.

Doležel, J., J. Macas and S. Lucretti, 1999 Flow analysis and sorting of plant chromosomes, pp. 5.3.1.–5.3.33. in *Current Protocols in Cytometry*, edited by J. P. Robinson, Z. Darzynkiewicz, P. N. Dean, L. G. Dressler, A. Orfao, P. S. Rabinovitch, C. C. Stewart, H. J. Tanke and L. L. Wheeless. John Wiley & Sons, New York.

Doležel, J., M. A. Lysák, M. Kubaláková, H. Šimková, J. Macas *et al.*, 2000 Sorting of plant chromosomes, in *Methods in Cell Biology: Flow Cytometry*, Ed. 3, edited by Z. Darzynkiewicz. Academic Press, San Diego (in press).

Endo, T. R., and B. S. Gill, 1996 The deletion stocks of common wheat. J. Hered. **87:** 295–307.

Ferguson-Smith, M. A., 1997 Genetic analysis by chromosome sorting and painting: phylogenetic and diagnostic applications. Eur. J. Hum. Genet. **5:** 253–265.

Friebe, B., and B. S. Gill, 1994 C-band polymorphism and structural rearrangements detected in common wheat (*Triticum aestivum*). Euphytica **78:** 1–5.

Fuchs, J., S. Joos, P. Lichter and I. Schubert, 1994 Localization of vicilin genes on field bean chromosome II by fluorescent in situ hybridization. J. Hered. **85:** 487–488.

Gamborg, O. L., and L. R. Wetter, 1975 *Plant Tissue Culture Methods.* National Research Council of Canada, Saskatoon.

Gill, B. S., 1987 Chromosome banding methods, standard chromosome nomenclature and applications in cytogenetic analysis, pp. 243–254 in *Wheat and Wheat Improvement*, Ed. 2, edited by E. G. Heyne. American Society of Agronomy, Madison, WI.

Gill, B. S., B. Friebe and T. R. Endo, 1991 Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat. Genome **34:** 830–839.

Gill, K. S., B. S. Gill, T. R. Endo and E. V. Boyko, 1996 Identification and high-density mapping of gene-rich regions in chromosome group of wheat. Genetics **143:** 1001–1012.

Gill, K. S., K. Arumuganathan and J. H. Lee, 1999 Isolating individual *wheat* (*Triticum aestivum*) chromosome arms by flow cytometric analysis of ditelosomic lines. Theor. Appl. Genet. **98:** 1248–1252.

Kubaláková, M., J. Macas and J. Doležel, 1997 Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PRINS. Theor. Appl. Genet. **94:** 758–763.

Lan, H., L. A. Shepel, J. D. Haag and M. N. Gould, 1999 Linkage mapping of rat chromosome 5 markers generated from chromosome-specific libraries. Mamm. Genome **10:** 687–691.

Lebo, R. V., 1982 Chromosome sorting and DNA sequence localization: a review. Cytometry **3:** 145–154.

Lee, J. H., K. Arumuganathan, S. M. Kaeppler, H. F. Kaeppler, and C. M. Papa, 1996 Cell synchronization and isolation of metaphase chromosomes from maize (*Zea mays* L.) root tips for flow cytometric analysis and sorting. Genome **39:** 697–703.

Lee, J. H., K. Arumuganathan, Y. Yen, S. Kaeppler, H. Kaeppler *et al.*, 1997 Root tip cell cycle synchronization and metaphase-chromosome isolation suitable for flow sorting in common wheat (*Triticum aestivum* L.). Genome **40:** 633–638.

Lucretti, S., J. Doležel, I. Schubert and J. Fuchs, 1993 Flow karyotyping and sorting of *Vicia faba* chromosomes. Theor. Appl. Genet. **85:** 665–672.

Lysák, M. A., J. Číhalíková, M. Kubaláková, H. Šimková, G. Künzel *et al.*, 1999 Flow karyotyping and sorting of mitotic chromosomes of barley (*Hordeum vulgare* L.). Chrom. Res. **7:** 431–444.

Macas, J., J. Doležel, S. Lucretti, U. Pich, A. Meister *et al.*, 1993 Localization of seed storage protein genes on flow-sorted field bean chromosomes. Chrom. Res. **1:** 107–115.

Macas, J., G. Gualberti, M. Nouzová, P. Samec, S. Lucretti *et al.*, 1996 Construction of chromosome-specific DNA libraries covering the whole genome of field bean (*Vicia faba* L.). Chrom. Res. **4:** 531–539.

McCormick, M. K., E. Campbell, L. Deaven and R. Moyzis, 1993 Low-frequency chimeric yeast artificial chromosome libraries

from flow-sorted human chromosomes 16 and 21. Proc. Natl. Acad. Sci. USA **90:** 1063–1067.

Nagaki, K., H. Tsujimoto and T. Sasakuma, 1998 Dynamics of tandem repetitive Afa-family sequences in triticeae, wheat-related species. J. Mol. Evol. **47:** 183–189.

Neumann, P., M. Lysák, J. Doležel and J. Macas, 1998 Isolation of chromosomes from *Pisum sativum* L. hairy root cultures and their analysis by flow cytometry. Plant Sci. **137:** 205–215.

Pan, W. H., A. Houben and R. Schlegel, 1993 Highly effective cell synchronization in plant roots by hydroxyurea and amiprophos-methyl or colchicine. Genome **36:** 387–390.

Pedersen, C., and P. Langridge, 1997 Identification of the entire chromosome complement of bread wheat by two-colour FISH. Genome **40:** 589–593.

Rayburn, A. L., and B. S. Gill, 1986 Molecular identification of the D-genome chromosome of wheat. J. Hered. **77:** 253–255.

Röder, M. S., V. Korzun, K. Wendehake, J. Plaschke, M. H. Tixier *et al.*, 1998 A microsatellite map of wheat. Genetics **149:** 2007–2023.

Schubert, I., J. Doležel, A. Houben, H. Schertan and G. Wanner, 1993 Refined examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. Chromosoma **102:** 96–101.

Schwarzacher, T., M. L. Wang, A. R. Leitch, N. Miller, G. Moore *et al.*, 1997 Flow cytometric analysis of the chromosomes and stability of a wheat cell-culture line. Theor. Appl. Genet. **94:** 91–97.

Sears, E. R., 1954 The aneuploids of common wheat. Mo. Agric. Exp. Stn. Res. Bull. **572:** 1–58.

Stein, N., N. Ponelies, T. Musket, M. McMullen and G. Weber, 1998 Chromosome micro-dissection and region-specific libraries from pachytene chromosomes of maize (*Zea mays* L.). Plant J. **13:** 281–289.

Telenius, H., N. P. Carter, C. E. Bebb, M. Nordenskjold, B. A. J. Ponder *et al.*, 1992 Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics **13:** 718–725.

Van Dilla, M. A., and L. L. Deaven, 1990 Construction of gene libraries for each human chromosome. Cytometry **11:** 208–218.

Wang, M. L., A. R. Leitch, T. Schwarzacher, J. S. Heslop-Harrison and G. Moore, 1992 Construction of a chromosome-enriched *Hpa*II library from flow-sorted wheat chromosomes. Nucleic Acids Res. **20:** 1897–1901.

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