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Flow cytometric analysis of the chromosomes and stability of a wheat cell-culture line

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Abstract A rapidly growing, long-term suspension culture derived from *Triticum aestivum* L. (wheat) was synchronized using hydroxyurea and colchicine, and a chromosome suspension with $2–3 \times 10^6$ chromosomes ml^{-1} was made. After staining with the DNA-specific fluorochromes Hoechst 33258 and Chromomycin A_3, univariate and bivariate flow-cytometry histograms showed 15 clearly resolved peaks corresponding to individual chromosome types or groups of chromosomes with similar DNA contents. The flow karyotype was closely similar to a histogram of DNA content measurements of Feulgen-stained chromosomes made by microdensitometry. We were able to show the stability of the flow karyotype of the cell line over a year, while a parallel subculture had a slightly different, stable, karyotype following different growth conditions. The data indicate that flow cytometric analysis of plant karyotypes enables accurate, statistically precise chromosome classification and karyotyping of cereals. There was little overlap between individual flow-histogram peaks, so the method is useful for flow sorting and the construction of chromosome specific-recombinant DNA libraries. Using bivariate analysis, the AT:GC ratio of all the chromosomes was remarkably similar, in striking contrast to mammalian flow karyotypes. We speculate about a fundamental difference in organization and homogenization of DNA sequences between chromosomes within mammalian and plant genomes.

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Introduction

The application of flow cytometry to suspensions of isolated chromosomes enables the DNA content of each individual chromosome to be measured. When thousands of chromosomes are measured, a frequency histogram can be made to show the number of chromosomes with any particular DNA content, and the individual chromosome 'peaks' can be classified (the flow-karyotyping procedure). Gray and Cram (1990) concisely described the advantages of flow cytometry for plant molecular cytogenetics: "The analysis and sorting of plant chromosomes is of considerable economic interest. As is the case for mammalian chromosomes, flow karyotyping and chromosomes sorting provides the opportunity for gene mapping and the construction of chromosome-specific libraries." After the first report of plant flow karyotyping appeared in 1984 (de Laat and Blaas 1984), technical difficulties, including the cell walls, low metaphase index and similarity of chromosomes in most species (see review of Dolezel et al. 1994), have limited the application of flow karyotyping in plants. Nevertheless, a few groups have since isolated plant chromosomes at sufficient concentration and purity to enable their analysis by flow cytometry (Conia et al. 1987, 1989; Arumuganathan et al. 1991; Lucretti et al. 1993; Veuskens et al. 1995), the generation of chromosome-specific DNA libraries (Wang et al. 1992; Macas et al. 1993), and amplification of DNA sequences from sorted chromosomes by PCR (Arumuganathan et al. 1993).

Many important plant species have complex genomes and large, similarly sized chromosomes. For example, bread wheat, *Triticum aestivum* L. (2n = 6x = 42, 4C DNA content 69.3 pg) and related Triticeae species have chromosomes with an average DNA content of some 100 Mb (20% of the human genome).

Karyotype analysis of wheat has been extremely valuable for genetics, cytogenetics and plant breeding (see Gupta and Tsuchiya 1991), but is labour intensive and difficult. Two groups have been able to identify wheat lines with rye chromosomes additions by flow cytometry (Bashir et al. 1993; Pfosser et al. 1995), the lines differing by about 2% in DNA content. When chromosomes can be isolated from any dividing plant material (Lucretti et al. 1993), flow cytogenetics can determine rapidly any karyotype and hence would be able to detect changes in cereal karyotype evolution in culture or breeding programmes. Targeted gene isolation from plant species with large genomes has proved difficult, but there is a major advantage in starting with a compartmentalized genome. Microdissection of chromosomes from spread metaphases has been used for this purpose (Sandery et al. 1991; Fukui et al. 1992; Jung et al. 1992; Schondelmaier et al. 1993). Flow cytometry and chromosome sorting offers many advantages in terms of the number of chromosomes available for cloning and analysis, its ease, the analytical data acquired simultaneously, and the size of the resultant clones. Much of the analysis of the human genome relies on libraries from flow-sorted chromosomes (Deaven et al. 1986; Cotter et al. 1989). In preliminary experiments, we have made a recombinant DNA library from a single, well-separated, chromosome peak from a wheat cell-culture line (Wang et al. 1992).

In the present work, we describe methods for chromosome isolation from a cell suspension culture of *T. aestivum* (TaKB1, Leitch et al. 1993), the generation of univariate and bivariate flow karyotypes using the fluorescent dyes Hoechst 33258 and Chromomycin A_3, the sorting of chromosomes from the line, the characterization of the content of the sorted material, and the use of flow karyotyping to analyse the chromosome changes occurring during cell culture.

Materials and methods

A *T. aestivum* L. suspension culture, TaKB1 (Leitch et al. 1993), was used for microdensitometry, chromosome isolation and flow karyotyping. TaKB1, having been maintained on agar plates and subcultured once every 2–3 weeks, was diluted 3:1 in fresh P10 medium (Kao 1977) twice per week and grown at 25°C on a shaking platform, giving a doubling time of 54 h. TaKB1b, derived from the same line as TaKB1, was kept growing on agar for a further 20 months and was then subcultured in liquid medium as described above. Cells were synchronized at metaphase using the methods described by Leitch et al. (1993). Briefly, hydroxyurea (Sigma) was added to 50 ml of cultured cells in medium to give a final concentration of 2.5 mM. The cells were incubated at 25°C for 25 h, washed, and allowed to recover in fresh medium at 25°C for 13 h before adding

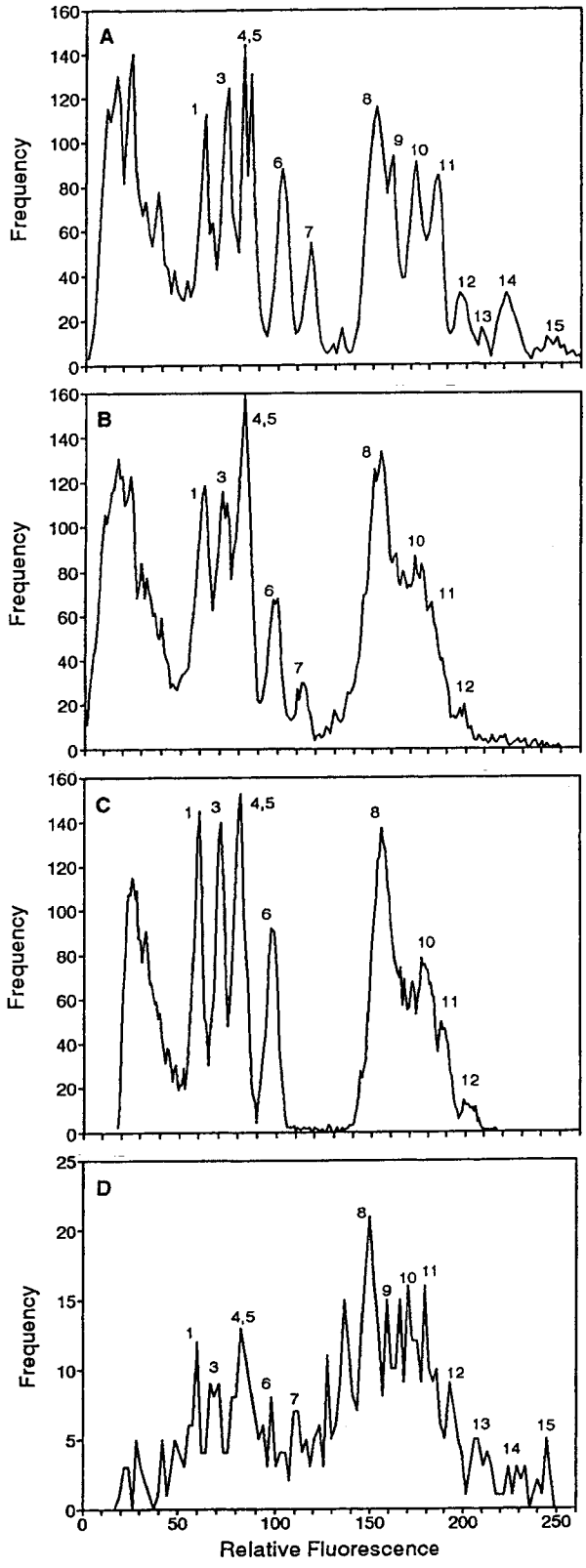


Fig. 1A–D Flow histograms of mitotic chromosomes from a long-term wheat suspension culture stained with Hoechst 33258 and Chromomycin A_3. Each univariate frequency histogram, for 50 000 events, shows the relative number of events (vertical axis) at each fluorescent intensity (horizontal axis) representing the sum of the two channels. **A, B** Histogram of the cell line TaKB1 made 3 months apart. **C** A histogram of TaKB1b made 18 months after **B** (photomultiplier voltages set to cut off peaks over 220 on this scale). **D** Frequency histogram of DNA content obtained from 650 randomly selected Feulgen-stained chromosomes from TaKB1 (2 weeks after **A**) measured by microdensitometry

0.05% (w/v) colchicine as a mitotic inhibitor. The colchicine treatment was carried out in the dark at 4°C while shaking gently. Cells were harvested after 16 h.

Sterile techniques were used until the chromosome-staining stages; four to six tubes were treated in parallel from two different sub-cultures. About 2 ml of the synchronized cell suspension was decanted into a Petri dish, drained, and washed for 1 min in 0.1 M EGTA [ethylene glycol-bis(beta-aminoethyl ether)]. After draining, cell walls were rapidly digested at 32°C for 8–10 min in 3% (w/v) cellulase 'Onozuka' RS, 0.5% (w/v) macerozyme R-10 (Yakult Pharmaceutical Industry), and 0.25% (w/v) pectolyase Y-23 (Seishin Pharmaceutical) in CPW salts (Frearson et al. 1973) containing 0.5 M mannitol. After digestion, protoplasts were gently centrifuged at 100 g for 3 min, drained, and the pellet re-suspended in 0.5 M mannitol. After 1 min incubation, protoplasts were re-sedimented at 100 g for 3 min and were re-suspended in 1 ml of ice cold, hypotonic, potassium chloride (75 mM) with polyamines (0.2 mM spermine, 0.5 mM spermidine) to stabilize the chromosomes (Sillar and Young 1981; Carter 1994a; Dolezel et al. 1994). Protoplasts were left on ice for about 15 min and chromosomes were liberated by adding 2 µl of Triton X-100, shearing the suspension through a Pasteur pipette 10–12 times, and vortexing for 5–10 s. The suspension was centrifuged at 100 g for 1 min to sediment the denser interphase nuclei and cellular debris. The upper nine tenths of the supernatant, containing chromosomes and some interphase nuclei, was used as the chromosome suspension. At several stages during the isolation procedure, particularly to monitor and check the metaphase index, enzyme digestion and chromosome liberation, 5–10 µl of the culture were put on a glass slide, stained with DAPI (4′, 6-diamidino-2-phenylindole) and examined under the fluorescent microscope.

The chromosomes suspension was stained 2 h before flow cytometry by adding Hoechst 33258 (final concentration 25 µg ml^{-1}), Chromomycin A_3 (50 µgml^{-1}) and $MgCl_2$ (2.5 mM). Aliquots from each parallel tube were examined in an epifluorescence microscope, and tubes with a high proportion and absolute number of single chromosomes was selected for cytometric analysis. About 15 min before flow cytometry, 12 mM sodium citrate and 30 mM sodium sulphite were added, and the suspension centrifuged at 50 g for 1 min. The supernatant chromosome suspension, including some interphase nuclei and nuclear debris, was analysed on the day of isolation.

The chromosome suspension was analysed using FACStar Plus flow cytometers (Becton Dickinson) fitted with two 5 W Spectra Physics 2020 lasers. The primary laser was turned to emit UV light at 351–364 nm (300 mW) to excite Hoechst 33258, and the secondary laser turned at 458 nm (300 mW) to excite Chromomycin A_3. Hoechst 33258 fluorescence was measured using a 390-nm long-pass and a 480-nm short-pass filter; Chromomycin A_3 fluorescence was measured through a 490-nm long-pass filter only. A 2″ focal length spherical lens was used for beam shaping. Data were acquired at 250 events per s and 50 000 events were measured in each analysis. The values in each channel of the Hoechst 33258 and Chromomycin A_3 fluorescence intensities were projected onto a diagonal "DNA line" running through the origin and summed to form the univariate frequency histograms, as described by Trask et al. (1990). Chromosomes were sorted into microcentrifuge tubes using gates placed around each peak; in most cases, two peaks were sorted simultaneously. Drops from the tubes were placed on a microscope slide, covered with a coverslip and examined by epifluorescence microscopy without further staining.

For quantitative measurements of the DNA contents of individual chromosomes by microdensitometry, cells of TaKB1 were fixed in three parts ethanol to one part glacial acetic acid, stained with Feulgen's reagent and spread on glass slides following the method of Bennett and Smith (1976). The absorbance of individual metaphase chromosomes or chromatids was measured at 565 nm using a Vickers M86 scanning microdensitometer with a spot size of 0.4 µm and a 100x, 1.25 NA objective lens.

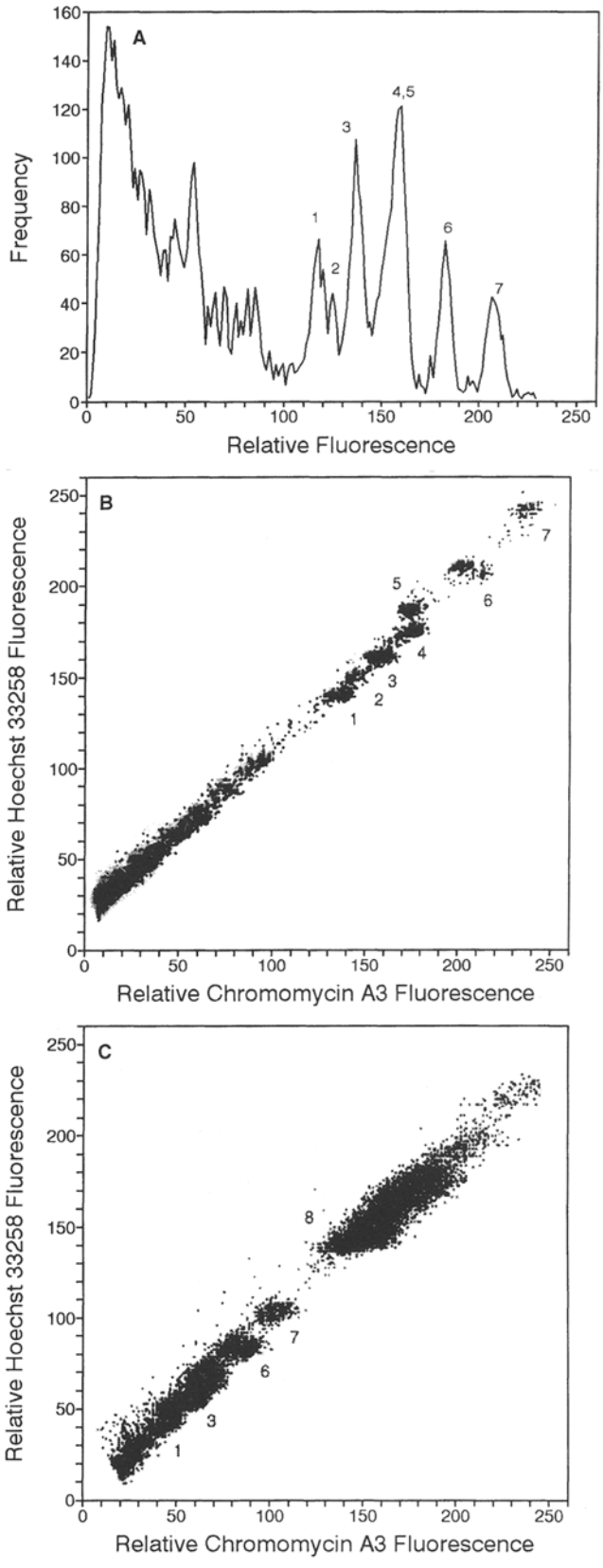


Fig. 2A A high-resolution flow histogram of TaKB1 (same chromosome suspension as used for Fig. 1A, but made with increased photomultiplier voltages) of peaks 1 to 7, where peak 2 can be resolved. **B** Bivariate flow karyotype (Corresponding to **A**) of TaKB1 showing relative fluorescent intensities of the two fluorechromes where the number of events is indicated by the density of dots. **C** Full bivariate flow karyotype of TaKB1 made 5 months after **A** and **B**

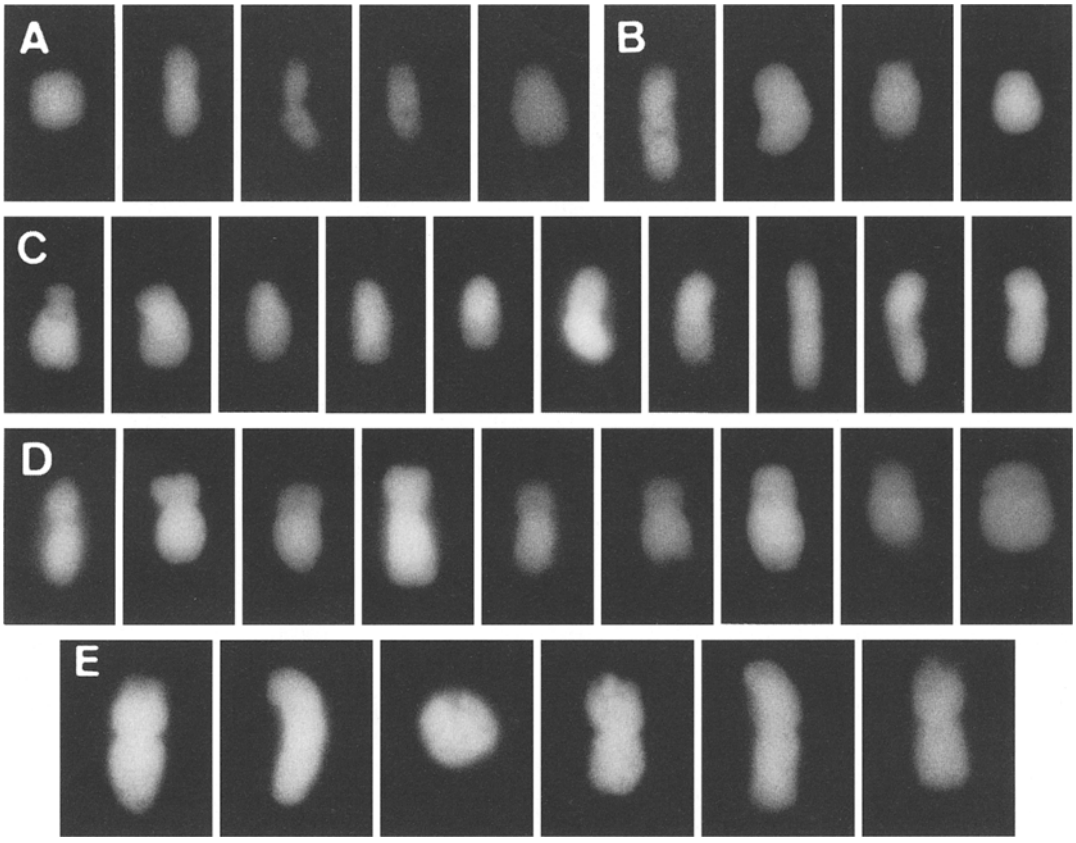
Results

The synchronization procedure using hydroxyurea and colchicine at 4°C gave a metaphase index of 30% from the TaKB1 wheat cell-suspension line (Leitch et al. 1993), and yielded typically 2.5×10^6 chromosomes ml^{-1}, which was sufficient to obtain flow histograms (see below). Of the particles seen by fluorescence microscopy in typical preparations used for flow cytometry, no more than 15% were clusters of chromosomes, broken nuclei, or broken chromosomes. The ratio of interphase nuclei to metaphase chromosomes was lower than 1:2, but could be improved by repeated centrifugation to separate chromosomes from nuclei. Occasional micronuclei were seen.

Figure 1A and B show univariate frequency histograms of chromosomes isolated from the cell line TaKB1, each for 50 000 events, with the number of events (vertical axis) occurring at each fluorescent intensity (horizontal axis; the sum of the two fluorescence channels Hoechst 33258 and Chromomycin A_3). The minimum peak height considered significant was taken to be about three-times greater than background levels. Fluorescence from a mycoplasma-like-organism, growing symbiotically in the cell-suspension culture (Chen et al. 1994), contributed to background fluorescence. Intact nuclei at G_1 in the suspension have a higher fluorescence than any of the peaks shown (see Leitch et al. 1993).

Fifteen major and distinct peaks were visible, each arising from a chromosome or chromosomes with a characteristic fluorescence intensity. The fluorescence channel number (horizontal axis) gives an indication of the relative chromosome size, which varied over a five-fold range. There were two groups of peaks separated by a gap where few particles were detected. Figure 1A has a lower coefficient of variation (about 2%) than Fig. 1B (about 4%) where the peaks are broader and less well defined. The reduction in the higher fluorescent peaks seen in Fig. 1B (peaks 13, 14 and 15) is probably caused by fragmentation of chromosomes during preparation. Further runs gave similar histograms over 16 months, indicating that the flow karyotype of the cell line is not changing substantially over that period. Figure 1C, made from TaKB1b transferred to liquid medium from agar some 20 months after TaKB1 (Fig. 1A and B), demonstrates that a single peak, number 7, is missing. Further analyses, over the next 6 weeks, gave the same flow karyotype, without peak 7. Figure 1D shows the distribution of DNA content from 650 individual chromosomes from TaKB1 (2 weeks after Fig. 1A) measured

Fig. 3A–E Photomicrographs of fluorescently stained flow-sorted chromosomes from selected peaks. **A** peak 4 is made of a sub-metacentric chromosome; **B** Peak 5, a metacentric chromosome with contamination from one (right) sub-metacentric chromosome of the type found in peak 4; **C** Peak 6, including two chromosome types, a sub-acrocentric (left) and a metacentric (right); **D** Peak 7, a sub-metacentric chromosome; **E** Peak 8, a metacentric chromosome and one micronucleus



by microdensitometry. Two major groups of peaks were visible with a six-fold size range. Peaks corresponding to those seen in the flow karyotypes (Fig. 1 A–C) are indicated by numbers. Chromatids were measured separately by microdensitometry, and all were in the lower group of peaks (nos. 1–5).

Analysis with higher photomultiplier voltages gave better resolution of the first group of peaks (of lower DNA content; Fig. 2A and B). Figure 2B shows a bivariate flow karyotype as a scatter plot, with the fluorescent intensities of the two fluorochromes on the X and Y axes, and the number of events indicated by the density of dots. All peaks are more clearly resolved than in the univariate histograms (Figs. 1 A–C and 2A); peaks 4 and 5 are separated in the vertical, Hoechst 33258 channel, although having similar Chromomycin A_3 fluorescences. A full bivariate histogram of TaKB1 is shown in Fig. 2 C indicating that the ratio of Chromomycin A_3: Hoechst 33258 fluorescence for all chromosomes of the complement, apart from minor exceptions (Fig. 2 B), is very similar.

Particles from selected peaks, and the region with lower fluorescence than any peak, were sorted and examined by epifluorescence microscopy. The low fluorescence region showed fibrous pieces of DNA and various unidentifiable, amorphous particles, while chromosomes of increasing size were present in sorts from peaks 1 to 8. The condensation of the chromosomes varied widely, and a few micronuclei were included. Chromosomes sorted from peak 4 (Fig. 3A) and peak 5 (Fig. 3B) are similar in size but show morphological differences. In the sorted materials, one chromosome of peak type 4 was seen in peak 5, so some contamination was present. Two types of chromosomes were observed in peak 6 (Fig. 3C), one metacentric and one acrocentric. Peak 7 (Fig. 3D) chromosomes were sub-metacentric. In peak 8 (Fig. 3E) large metacentric chromosomes were sorted, along with a micronucleus.

Discussion

The clear peaks shown in the flow karyotypes (Figs. 1, 2) and fluorescence microscopy of sorted particles (Fig. 3) indicate that many intact chromosomes were analysed. Wheat chromosomes are very large, break easily and stick together, but a technique closely following that used for mammalian chromosome preparation (Carter 1994a) gave a chromosome suspension suited for flow karyotyping and chromosome sorting. As reported elsewhere (Griesbach et al. 1982; see Dolezel et al. 1994), the plant chromosome-isolation method combining protoplast techniques (Conia et al. 1987, 1989) with the polyamine procedure for mammalian chromosomes (Blumenthal et al. 1979) gives stable and mostly complete chromosomes. We used a colchicine treatment at 4°C; colchicine treatment alone leads to the splitting of chromosomes and the formation of micronuclei (Verhoeven et al. 1990), but the combination with cold-treatment accumulates heavily condensed, but intact, chromosomes that do not separate frequently into individual chromatids (Fig. 3). The most critical steps during the isolation were the time of enzyme treatment, the hypotonic treatment, the incubation with Triton X-100, and the duration of mechanical liberation of the chromosomes by vortexing. The suspension was enriched in chromosomes by repeated centrifugation. The addition of sodium sulphite shortly before sorting to stabilize the chromosomes was also advantageous. Dolezel et al. (1994) reviewed in detail the requirements for plant flow cytometry and emphasized that the quality of the flow karyotype largely depends on the quality and purity of the chromosome preparation. Careful methods, and starting with large numbers of chromosomes, help to obtain a final suspension with many separate and intact chromosomes that is free of large (e.g. intact or broken nuclei, micronuclei and chromosome clumps) and small (e.g. broken chromosomes or split chromatids) particles that obscure distinct peaks, increase the spread of peaks and background, and tend to block the cytometer nozzle.

The flow karyotypes showed 15 discrete peaks, which were reproducible between runs and over many months (Figs. 1 A, B; 2 C), each representing the fluorescence of one chromosome, or a group of chromosomes, with a particular DNA content. Theoretical flow karyotypes of plant chromosomes can be modelled from light-microscope karyotypes using chromosome size measurements and estimated standard deviations (Conia et al. 1989; Dolezel 1991). Such simulations have been used to interpret flow karyotypes of *Lycopersicon* (Arumuganathan et al. 1991), *Nicotiana* (Conia et al. 1989), and *Petunia* (Conia et al. 1987) cell lines, as well as *Vicia faba* root tips (Lucretti et al. 1993). However, cultured material often shows chromosome rearrangements and the light-microscope karyotype of TaKB1 has changed in culture from the normal wheat karyotype so that cells now have variable chromosome numbers (14–25) and a total of about 27 distinct chromosome types (Leitch et al. 1993). Since comparisons with a theoretical flow karyotype of TaKB1 were impossible, the flow karyotype was compared with the DNA content of chromosomes measured by microdensitometry and the two analyses correlated well. The five-fold range in fluorescent intensity of the 15 major peaks in the flow karyotype (Fig. 1A) is similar to, but less than, the range of peaks found by microdensitometry (Fig. 1 D). More chromosomes with a low DNA content are resolved by microdensitometry which were obscured by broken chromosomes or nuclear debris in the flow karyotypes. Larger peaks were also less clear in the flow karyotype, suggesting the loss or breakage of large chromosomes during preparation. A reduction of the large chromosomes peaks in the flow karyotype of *V. faba* was also reported by Lucretti et al. (1993).

The stability of the flow karyotype of the TaKB1 line is surprising considering that cytogenetic studies showed extreme karyotype variation from cell to cell (Leitch et al. 1993), which is typical of plant cell cultures showing somaclonal variation (Lee and Phillips 1988).

The stability of the flow karyotype over more than 1 year suggests that cell lineages with new chromosome rearrangements are not proliferating in the culture to such an extent that the culture as a whole is altering. However, in TaKB1b, which was brought to liquid medium at a different time than TaKB1, the flow karyotype missed one particular chromosome type, but also remained stable. Presumably, genetic drift and the founder effect, as described for plant populations, allows cells with different karyotypes to be established randomly when the cell culture goes through a physiological change, e.g. transfer from a semi-solid to liquid medium.

Pools or amplification products from flow-sorted mammalian chromosomes are widely used for chromosome 'painting', where the labelled chromosome product hybridizes in situ to its chromosome-of-origin in chromosome spreads. Fuchs et al. (1996) suggest that chromosome painting is not possible in plants using clones derived from microdissected or flow-sorted chromosomes, at least using hybridization methods similar to those employed for humans. The similarity of the bulk DNA composition of all wheat chromosomes revealed by the bivariate flow karyotype (Fig. 2B and C), showing similar ratios of Hoechst 33258 fluorescence (enhanced by a higher proportion of adenine and thymine, AT, bases) to Chromomycin A_3 fluorescence (guanine/cytosine, GC enhanced), supports the suggestion that use of whole chromosome-derived probes may not be successful for chromosome painting in plants. All mammalian flow karyotypes presented so far (Carter 1994b; Langford et al. 1996) show substantial variation between most individual chromosomes in GC:AT ratio (i.e. a wide spread of clusters of dots in karyotypes such as Fig. 2B), while the plant karyotypes analysed by bivariate analysis, wheat (Fig. 2B and C) and tomato (Arumaganathan et al. 1991), show all chromosomes of the complement with similar fluorescence from the two stains. Hence all the chromosomes within the plant species analysed have a similar base-pair ratio, which we suggest indicates extensive homogenization of sequences between chromosomes within a genome. During cereal evolution, a few inter-chromosomal reciprocal translocation, some of relatively recent origin (< 50000 years), have been detected by mapping methods (Devos et al. 1993), and further translocations might be present in the cell line used by us. However, translocations alone cannot account for the level of homogenization seen; there are not the large number that would be required for homogenization. The greater part of the cereal genome is composed of repetitive DNA sequences, including retroelements and sequences arranged in long arrays of tandem repeats. Although the sequences differ in AT:GC ratio, many are present on most or all chromosomes (e.g. retroelements: Schmidt et al. 1995; Pearce et al. 1996; tandem repeats: Galasso et al. 1995; Vershinin et al. 1995). When human chromosomes carrying small deletions are flow sorted, amplified, labelled and used as probes in situ on normal chromosome spreads, the deletion of the flow-sorted chromosome is visualized by an unlabelled region on the normal chromosome spread (Carter et al. 1992), indicating that homogenization of sequences does not occur even along one chromosome in mammals. The difference between mammalian bivariate flow karyotypes and plant flow karyotypes is remarkable, perhaps indicating a fundamental difference in the predominant modes of sequence dispersion and genome evolution between chromosomes in the two taxa.

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