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Isolating individual wheat (*Triticum aestivum*) chromosome arms by flow cytometric analysis of ditelosomic lines

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Abstract We are reporting the successful isolation of wheat chromosome arm 1DS by flow cytometry. A chromosome suspension was prepared for the 1DS ditelosomic line and the normal 'Chinese Spring' (CS) by chopping 2-day-old root tip meristems, synchronized by hydroxyurea, in HEPES-magnesium sulfate buffer containing propidium iodide. Chromosomes were analyzed and sorted with a FACS Vantage flow cytometer and cell sorter. An extra peak was observed in the flow karyotype of the ditelosomic line that was absent in 'CS'. The estimated size of chromosomes from the extra peak matched with the expected size of chromosome 1DS. Chromosomes from the putative 1DS peak were analyzed by both fluorescent microscopy and N-banding analysis. A total of 571 chromosomes from two separate experiments were analyzed, and all were observed to be telosomics except for 2 which were broken. About 82% of these telosomics showed the diagnostic N-band of 1DS, the remaining were unbanded and are probably also 1DS. This strategy can also be used to sort other wheat arms.

Key words Wheat · Chromosome sorting · Chromosome isolation · Aneuploids · *Triticum aestivum*

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Introduction

The haploid bread wheat genome of 16 million kb of DNA is packaged into 21 chromosomes. An effective way to reduce the complexity of a genome is to isolate and manipulate individual chromosomes/arms. Flow sorting has been used to individually sort all of the human chromosomes. Flow-sorted human chromosomes have been used to produce chromosome-specific phage (Fuscoe et al. 1986), plasmid (Collins et al. 1991), cosmid (Weber et al. 1994; Wood et al. 1992), Fosmid (Kim et al. 1995a) and YAC (McCormick et al. 1989, 1993a,b) libraries. Sorted chromosomes have also been used to develop 'chromosome painting' probes (Carter 1994), to screen various cDNA libraries in order to select chromosome-specific expressed sequences (de Fatima Bonaldo et al. 1994) and to identify chromosome-specific BAC clones (Kim et al. 1995b).

In plants, flow sorting karyographs have been reported in *Haplopappus gracilis*, *Petunia hybrida*, *Lycopersicon esculentum*, *L. pennellii*, *Triticum aestivum*, *Vicia faba*, *Pisum sativum* and *Zea mays* (De Laat and Blass 1984; Conia et al. 1987; Arumuganathan et al. 1991; Lucretti et al. 1993; Gualberti et al. 1996; Lee et al. 1997a). However, there is only one report of a pure single chromosome preparation of tomato chromosome 2, which was made possible only by bivariate analysis (Arumuganathan et al. 1994). For most plant species, flow-sorted chromosome fractions contain a mixture of chromosomes because of the lack of significant variation in chromosome size. In wheat, Lee et al. (1997b) separated 21 chromosomes into three major fractions by flow cytometry.

A wealth of aneuploid stocks are available in wheat which are invaluable for both genetic and molecular genetic studies (Sears 1954; Endo and Gill 1996). Among these, ditelosomic lines (possessing 20 pairs of normal wheat chromosomes and one pair of arms instead of the whole chromosome) are available for all 42 arms except for 7DS (Sears 1954). These ditelosomic

lines are excellent material for chromosome sorting as most of the chromosome arms are significantly shorter than the shortest wheat chromosome. The objective of the study reported here, therefore, was to explore the possibility of using ditelosomic lines to isolate individual wheat chromosome arms.

Materials and methods

Plant material

The ditelosomic 1DS line of wheat possesses the normal chromosome complement of wheat cultivar 'Chinese Spring' except for chromosome arm 1DL, which is absent. The ditelosomic line was originally developed by Dr. E.R. Sears (1954) and is being maintained by the Wheat Genetics Resource Center (WGRC), Manhattan, Kansas. Dr. B.S. Gill, Director of WGRC, generously provided seeds for the ditelosomic line and cv 'Chinese Spring'.

Chromosome preparation

The methods for cell synchronization and chromosome preparation and sorting were essentially as described by Lee et al. (1997a). Briefly, wheat seeds were germinated on a stack of wet filter papers for 2 days. Seedlings with 0.8- to 1.0-cm-long primary roots were selected and transferred onto filter papers (in petri dishes) soaked in 1.25 m*M* hydroxyurea solution. After 16 h, seedlings were washed in distilled water three times and incubated for 2 h on a filter paper soaked in hydroxyurea-free Hoagland's solution (Sigma). Seedlings were then incubated for 4 h on filter papers soaked in 1 μ*M* trifluralin. All incubations were done at room temperature (approx. 25°C) in the dark.

Root tips (approx. 1.5 mm) excised from synchronized seedlings were finely chopped in HEPES-magnesium sulfate chromosome isolation buffer [10 m*M* $MgSO_4.7H_2O$, 50 m*M* KCl, 5 m*M* HEPES buffer, 3 m*M* dithiothreitol (DTT) and 0.25% Triton X-100] with a sharp scalpel to release metaphase chromosomes from dividing cells. The suspension was allowed to pass through a 30-μm pore size mesh, and the chromosomes were stained with propidium iodide.

Flow cytometric analysis and sorting

Chromosome suspensions were analyzed with a FACS Vantage flow cytometer and cell sorter system (Becton Dickinson) at the Cell Analysis laboratory, Center for Biotechnology, University of Nebraska-Lincoln. Sorted particles from each peak were analyzed under a fluorescent microscope to identify chromosome containing fractions. Contents of each peak were sorted directly onto a black nitrocellulose filter (Millipore type AA, pore size 0.8 μm). The nitrocellulose filter was placed on a glass slide and analyzed under a fluorescent microscope using an appropriate filter for propidium iodide.

Chromosome banding analysis

Chromosomes from the putative 1DS peak were analyzed by N-banding analysis. Chromosomes were sorted directly into a microfuge tube containing an EtOH: acetic acid solution (3 : 1) in order to fix the chromosomes. Chromosomes were centrifuged at 10 000 rpm for 1 min in an Eppendorf centrifuge and resuspended in 5 μl of 1% acetocarmine prepared in 45% acetic acid. Chromosomes were placed on a glass slide under a cover slip. The slides were kept at −70°C for at least 2 h or until ready for N-banding analysis. The N-banding procedure was according to Endo and Gill (1984) except that the Giemsa staining time was less than half that for the regular root-tip squash preparations. Special care was taken during washing steps so as not to lose the chromosomes. Because of the smaller size of the 1DS telosomic chromosome and presence of debris, the slides were analyzed at 1000× magnification after staining with Giemsa.

Results

The method of root-tip cell synchronization followed by release of chromosomes by chopping in HEPES-magnesium sulfate buffer yielded a large number of intact chromosomes. Figure 1a shows mitotic metaphase chromosomes from a squashed root tip of 1DS. The telosomic chromosomes (marked by arrows) in Fig. 1a are much smaller than the remaining chromosomes. Chromosomes from ten such dividing cells were analyzed for their morphology, and no broken chromosome was observed. To check if the chopping method or chromosome isolation buffer damaged chromosome structure or morphology, we analyzed the chromosome suspension after filtration (see Materials and methods) under a microscope. Only intact chromosomes with good morphology were observed along with some nuclei. Again, no broken chromosome was observed.

From the root-tip squash preparations, the mitotic index was calculated for the 1DS ditelosomic line as a percentage of cells arrested in metaphase. This index was estimated to be 61%. Since not all chromosomes arrested in metaphase are released into the buffer by the chopping method, we wanted to estimate the number of chromosomes obtained from each seed. Twenty seeds of 'Chinese Spring' were germinated and synchronized, as described above. The chromosome suspension was analyzed by the cell sorter, and the number of particles present in the chromosome containing fractions (Fig. 2) was counted. A total of 82 000 chromosomes were recovered from 20 seedlings. Therefore, the current method yielded 4100 chromosomes per seed (each seed produces three root tips).

The flow karyotype of cv 'Chinese Spring' is given in Fig. 2a. Contents of all peaks are marked on the figure and are according to Lee et al. (1997a). All 21 chromosomes of wheat were sorted into three major peaks. The contents of one of the three peaks, expected to contain smaller wheat chromosomes, were sorted directly onto a black nitrocellulose filter and observed under a fluorescent microscope. Only normal metaphase chromosomes were observed (Fig. 1b). There were no broken chromosomes or telosomics. Centromeres of the chromosomes were clearly visible (some are marked by arrows). Morphology of the chromosomes was preserved. The flow karyotype of the 1DS ditelosomic line was similar to that of normal 'Chinese Spring' except

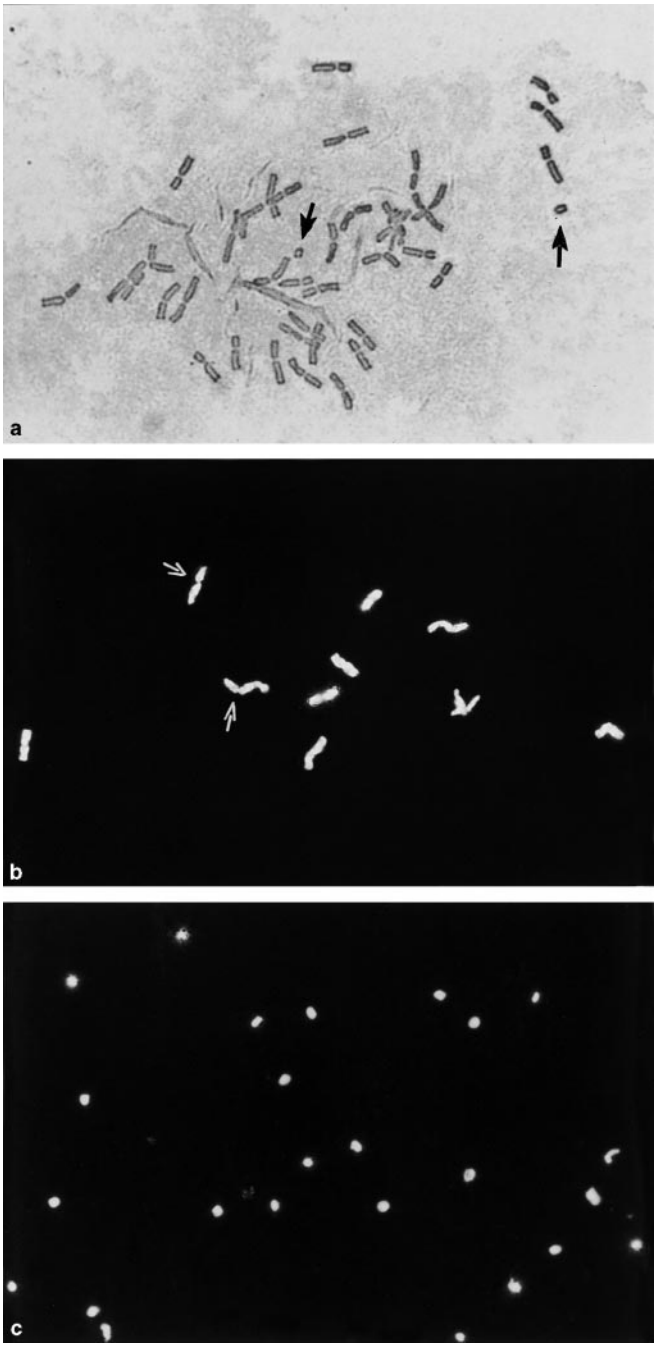

Fig. 1a–c **a** Mitotic metaphase root-tip chromosomes of the ditelosomic 1DS line of wheat. Seeds of ditelosomic 1DS line were germinated and synchronized as described in Materials and methods section. Root-tip cells were squashed and stained with modified carbol fuchsin solution (Law et al. 1982). The 1DS telosomics are marked by *arrows*. **b, c** Fluorescent microscopic analysis of various chromosome-containing peaks. Contents of the peaks were placed on a black nitrocellulose paper (Materials and methods) that was then placed on a glass slide, stained with propidium iodide and observed under a microscope. **b** Chromosomes from one of the three chromosome-containing peaks present in normal 'Chinese Spring'. The peak expected to contain the smallest chromosomes of 'CS' was used. Only intact chromosomes were observed, no telosomics or broken chromosomes. Centromeres of all the chromosomes are visible, and a few are marked by *arrows*. **c** The contents of the putative 1DS-containing peak that was present in the 1DS telosomic line but absent in normal 'Chinese Spring'

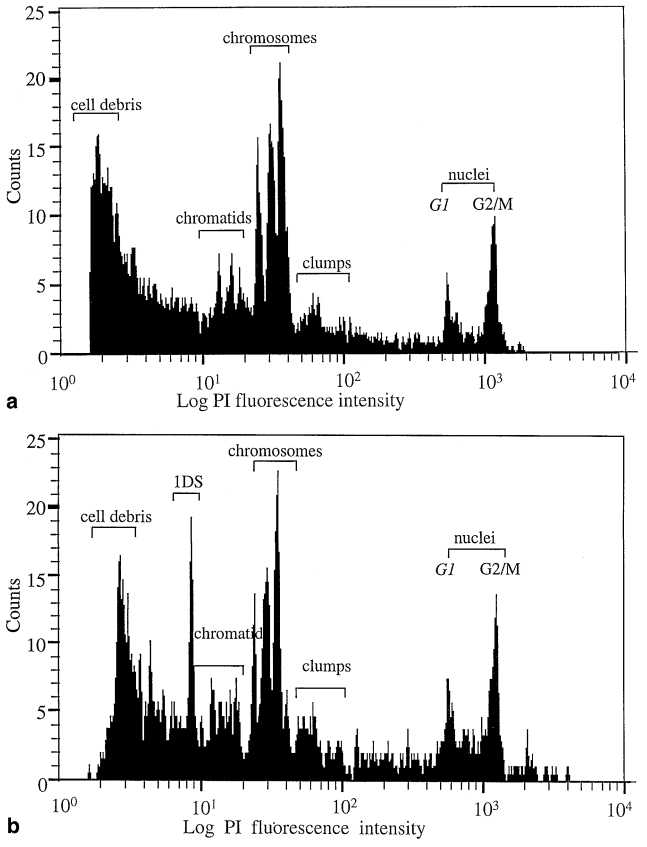

Fig. 2a, b Univariate flow karyotypes of normal 'Chinese Spring' wheat (**a**) and the 1DS ditelosomic line (**b**) generated from flow cytometric analysis of propidium iodide-stained chromosome suspensions. Note an extra peak in the 1DS ditelosomic line that is not present in normal 'Chinese Spring' wheat

that an extra peak was observed for the former (Fig. 2b). The peak was sharp with a narrow base, suggesting that the chromosomes of the peak were about the same size with little variation among their DNA content. The contents of the extra peak were sorted onto a black nitrocellulose filter and observed under a microscope. As expected, only small, telosomic chromosomes were observed (Fig. 1c). All the chromosomes appeared to be of the same size.

The size of the chromosome 1DS arm at mitotic metaphase is 3.13 μm compared to the 235 μm of the combined 21 wheat chromosomes (Gill et al. 1991). Therefore, chromosome arm 1DS is about 1/75 of the whole wheat genome. The haploid wheat genome is 16 million kb in size, and the 1DS chromosome is expected to be about 213 Mb. To check if the size of the chromosomes from the putative 1DS peak matched that of the expected size based on cytogenetic measurements, we estimated the DNA content of the putative 1DS chromosomes by comparing it with that of wheat G1 nuclei, as described by Lee et al. (1997b). Briefly, using wheat G1 nuclei (Fig. 2b) as a control, the DNA content of a chromosome peak is calculated using the

Table 1 N-banding analysis of the chromosomes from the putative 1DS peak

	Telosomic chromosomes		Non-1DS bands	Broken chromosomes	Total
	1DS bands	Unbanded			
Experiment 1	176	53	0	0	229
Experiment 2	292	48	0	2	342
Total	468	101	0	2	571

following equation:

$$\frac{\text{Relative mean fluorescence of the unknown chromosome peak}}{\text{Relative mean fluorescence of wheat G1 nuclei peak}} \times 2\text{C DNA content of wheat}$$

Following this method, the estimated DNA content of telosomic chromosomes from the putative 1DS peak was 220 Mb, about 1/72 of the total wheat genome.

To confirm the purity and identity of the sorted chromosomes, we analyzed putative 1DS chromosomes from the extra peak by N-banding analysis. Chromosome 1DS possesses a diagnostic N-band at a sub-telomeric location (Gill et al. 1991). Results of the banding analysis from two separate experiments are summarized in Table 1. A total of 571 chromosomes were analyzed. Except for 2 chromosomes that appeared to be broken, all of the chromosomes were telosomics. Out of these, 468 chromosomes showed the diagnostic N-band of chromosome 1DS. The remaining 103, including the 2 broken chromosomes, did not show any N-band. It is likely that most of these unbanded chromosomes are also chromosome 1DS because of the comparable small size and a typical telosomic chromosome morphology. With our technique of N-banding, some 1DS telosomic chromosomes from the metaphase spreads of ditelosomic lines also did not show the diagnostic band.

Discussion

Isolated chromosomes/arms have many applications in the molecular characterization of plant genomes including the production of chromosome- or arm-specific libraries, the development of chromosome-painting probes, the study of chromosome architecture, rapid mapping of expressed sequences and physical mapping. The main problem with sorting plant chromosomes is the narrow size range among their chromosomes. We postulated that ditelosomic lines in wheat would be useful in chromosome sorting with regard to bypassing this problem of a narrow chromosome size range. The size of any telosomic chromosome is significantly smaller than that of the smallest wheat chromosome. Using the ditelosomic line for 1DS arm as an example, we have shown here that wheat chromosome arms can be successfully sorted from their corresponding ditelosomic lines.

The purity of the sorted chromosomes was very high. Most of the 101 unbanded telosomics observed during N-banding analysis were most likely chromosome 1DS. All the telosomics appeared to be of the same size as 1DS and either showed the dignostic band for 1DS or were unbanded. Of the 21 wheat chromosomes, 16 can be identified by their unique N-banding pattern (Gill et al. 1991). The telosomics were not generated by chromosome breaks because breakages were not observed during sorting of normal CS.

The approach used in this study to isolate individual wheat chromosome arms can be extended to other chromosomes as well. For uses such as developing chromosome arm-specific libraries, 'chromosome-painting' probes, and fractionation of cDNA libraries into arm-specific pools, the sorting of only 14 arms, corresponding to the 14 wheat homoeologous group arms, should be adequate to represent the entire wheat genome. For complex gene expression studies such as dosage-dependent gene action and to study the concerted functioning of three copies of the wheat genes, the sorting of all wheat chromosome arms may be required. Except for 7DS, all 42 wheat arms are represented either as ditelosomic lines or as ditelo-monotelosomic lines (Friebe et al. 1996). Chromosomes varying by 10% in their size can be separated by flow cytometer. The smallest chromosome of the wheat genome is chromosome 1D which is 8.4 μm in size. Except for 5BL and 3BL, all wheat chromosome arms should be separable from other wheat chromosomes because the size difference is more than 10%.

Another important utility of sorted chromosomes is to generate chromosome-painting probes for molecular cytogenetics experiments such as the tracking of complex chromosomal regions and aberrations and establishing the homoeology of wheat with other Triticeae species including some lesser studied grasses. To develop a chromosome/arm-specific probe, we can use DNA

from sorted chromosomes directly as a probe or it can be amplified by degenerate oligonucleotide-primed PCR (DOP-PCR) (Telenius et al. 1992a,b).

The most important use of the sorted chromosomes we foresee is in functional genomics by using sorted chromosomes as probes to map expressed sequences to their respective chromosome arms even before these are isolated and characterized. Such a strategy was used to make 'expressed sequence tag' (EST) maps in humans where chromosome-specific cDNA clones were selected using sorted chromosomes as probes, sequenced and then converted into ESTs (de Fatima Bonaldo et al. 1994). In wheat, this approach can be taken one step further by mapping the cDNAs to chromosome arms rather than to whole chromosomes as in humans. The arm-specific cDNAs can be further mapped to their respective chromosomal regions using the array of deletion lines generated by Endo and Gill (1996).

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