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Construction of a chromosome-enriched HpaII library from flow-sorted wheat chromosomes

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ABSTRACT

We report here the first successful generation of a chromosome-enriched library from flow sorted plant chromosomes. Chromosomes with a characteristic DNA content (a peak) were sorted from a synchronized cell culture (TaKB1, derived from *Triticum aestivum*). A HpaII library was constructed from the sorted chromosomes and half of the cloned DNA sequences analysed are unique or low copy. Approximately half of these sequences when used as probes detect sequences on wheat chromosome 4A. The generation and analysis of the chromosome library is described in detail and the prospects of using flow-sorted plant chromosomes discussed.

INTRODUCTION

Wheat (*Triticum aestivum*, 2n=6x=42) accounts for more than 20% of the world's food supply. The construction of genetic maps of wheat and the subsequent use of characterized markers (RFLPs) as aids to help score valuable traits in plant breeding is progressing (1,2). The production of high density RFLP maps of the small grain cereals (wheat, barley and rye) would define more precisely map locations of genes controlling agronomically important traits. This would allow the isolation of genes determining these traits by chromosome walking (positional cloning). However these cereal species possess relatively large genomes. For example, the three genomes (A,B,D) of hexaploid wheat are 17×10^9 bp (in total per 1C nucleus) in size. So the average chromosome of wheat is approximately 0.8×10^9 bp or a quarter the size of the human genome (3). Clearly the ability to generate probes to specific chromosomes would be a major advance in plant and agricultural sciences.

The first human chromosomes were sorted in 1975 (4) and led to the generation of a chromosome-specific library in 1981 (5). Sorted chromosomes have been used to generate chromosome-specific libraries which are important tools in the human gene isolation programme and as a source of material for various other techniques (6). Although there have been reports of flow sorting plant chromosomes, from *Haplopappus gracillis* (2n=4) in 1984 (7) and tomato chromosomes (2n=24) in 1991 (8) as yet no chromosome-enriched library has been generated from sorted plant chromosomes. Therefore it is unclear whether the peaks in these plant flow karyotypes arise from different chromosomes. The flow sorting has been performed on plant chromosomes isolated from cell line material because the cells in culture achieve a higher mitotic index than those in root tips after chemical treatment, allowing more chromosomes to be isolated for sorting. Because plant cell culture lines may have an abnormal karyotype, libraries to chromosomes sorted from cell lines may not be enriched for clones mapping to a specific chromosome. We report here the generation of the first plant chromosome-enriched library built from sorted chromosomes isolated from a wheat cell line.

METHODS

Accumulating metaphases

To accumulate a higher metaphase index, the methods described by Leitch et al. (9) were used. Cells of a derivative of *Triticum aestivum*, TaKB1 (cell suspension culture) were subcultured every 3–4 days in P_{10} medium. Firstly, the cultured cells (50 ml) were incubated on a shaking platform in 2.5 mM hydroxyurea (Sigma) at 25°C for 25 hours to be synchronized at the boundary of G1/S. Secondly, the synchronized cells were washed with MS plus 3% (w/v) sucrose for 3 times and cultured back in P_{10} medium for 13 hours at 25°C to be recovered. Thirdly, the recovered cells were incubated in 0.05% (w/v) colchicine in the dark at 4°C with shaking for 18–20 hours to be accumulated at metaphase. Finally, the cells were harvested from the cell suspension.

Preparing protoplasts and chromosome suspension

To prepare protoplasts, the harvested cells from about 2 ml of the cell suspension were briefly washed in 0.1 M EGTA, and then rapidly digested for 8 min at 32°C in 3 ml of 3% (w/v) cellulase 'Onozuka' RS, 0.5% (w/v) macerozyme R-10 (Yakult Phamaceutical Industry), and 0.25% (w/v) pectolyase Y-23 (Seishin Pharmaceutical) dissolved in CPW salts (10) plus 0.5 M mannitol as an osmoticum. After digestion, protoplasts were centrifuged at 100 g for 3 min and the pellet resuspended in 0.5 M mannitol. After 1 min incubation, protoplasts were sedimented (100 g for 3 min).

To prepare chromosome suspension, the sedimented protoplasts were quickly resuspended in 1 ml of ice cold hypotonic,

potassium chloride (75 mM to swell the protoplasts), with polyamines (0.2 mM spermine, 0.5 mM spermidine to stabilize chromosomes) for about 15 min on ice. The chromosomes were liberated by adding 2 µl of Triton X-100, shearing the protoplasts through a Pasteur pipette twelve times, and vortexing for 5 sec. The suspension was centrifuged at 100 g for 1 min and the upper nine tenths of the supernatant, containing chromosomes and some interphase nuclei, was used as the chromosome suspension. The chromosome suspension was stained with Hoechst 33258 (25 µg /ml) and Chromomycin A_3 (50 µg /ml), and stabilized with $MgCl_2$ (2.5 mM) two hours before flow cytometry. About 15 min before flow cytometry, sodium citrate (12 mM) and sodium sulphate (30 mM) were added to the chromosome suspension.

Chromosome sorting and chromosomal DNA extraction

100,000 chromosomes were sorted from one peak (Fig.1, arrowed peak) on a FACStar Plus flow cytometer over 5 hours. The sorted chromosomes were pelleted by centrifugation at 13,000 rpm for 40 min at 4°C and the pellet was dried at RT for 30 min. The DNA extraction from the chromosomes followed a DNA isolation protocol from blood with minor changes (11). The pellet was vigorously resuspended in 35 µl ice cold Buffer B (5 mM sodium citrate, 20 mM NaCl, pH6.5). 5 µl of 4 µg/µl pronase E and 4 µl Buffer C (1.5% Sarkosyl, 20 mM EDTA, 20 mM Trizma base, pH 8.5) were added, vortexing after each addition. The suspension was then incubated at 45°C overnight.

To precipitate DNA, 40 µl TE (10 mM Tris HCl, 1.0 mM EDTA, pH 7.5), 10 µl 7.5 M ammonium acetate and 350 µl cold ethanol were added, mixing by inversion at each stage. The DNA was precipitated at −20°C overnight. The DNA was pelleted by centrifugation at 13,000 rpm for 30 min at 4°C . The DNA was washed with 70% cold ethanol twice, dried at RT and then resuspended in 5 µl TE.

Chromosomal DNA digestion

The chromosomal DNA was digested with HpaII overnight in NBL reaction buffer (Northumbria Biologicals Limited). After digestion, the DNA fragments were extracted by 20 µl phenol/chloroform/isoamylalcohol (25:24:1), precipitated by ethanol, dried for 1 hour at RT and dissolved in 5 µl TE.

Ligation and transformation

About 20 ng HpaII digested DNA extracted from either sorted or unsorted chromosomes was ligated into AccI digested pUBs1

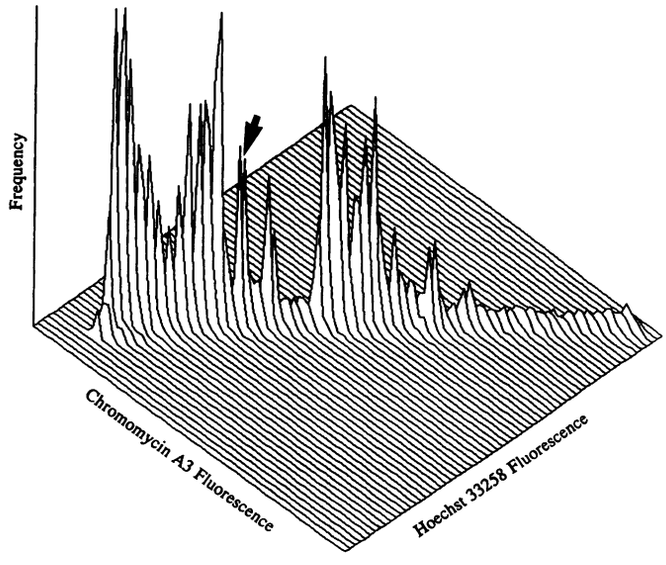

Fig.1. The bivariate flow karyotype of the TaKB1 cell line derived from *Triticum aestivum*. The arrow indicates the sorted peak. 100,000 chromosomes within this peak were collected for further analysis.

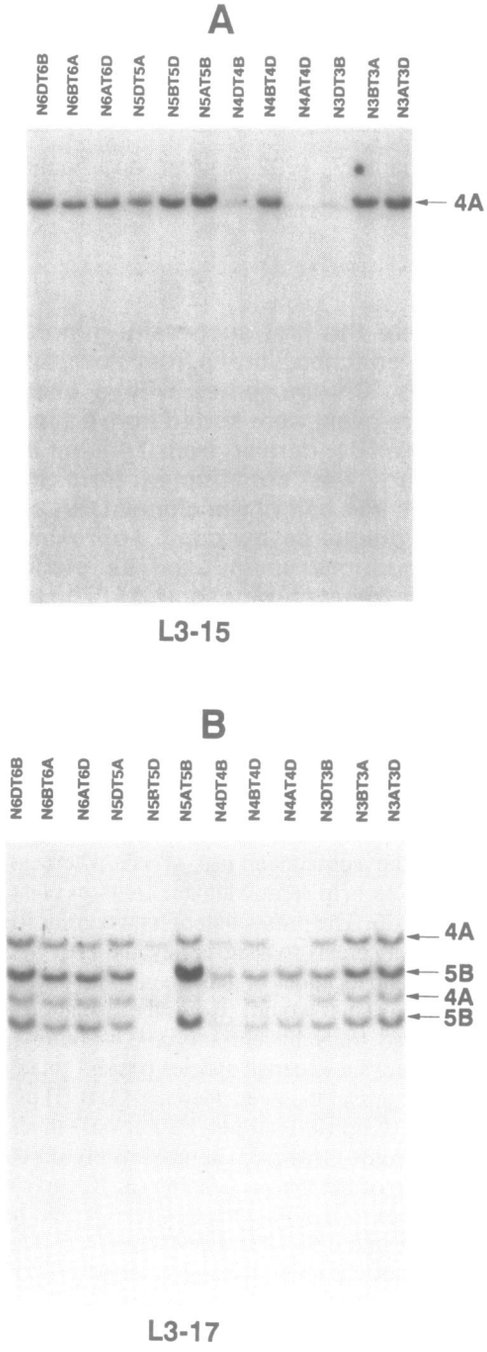

Fig.2. Location of HpaII clones from sorted chromosomal DNA. Southern blots of EcoRI-digested from DNA nulli-tetrasomic lines were probed with L3-15 (A) and L3-17 (B). The nomenclature N4AT4D indicates that the DNA has been isolated from a line in which chromosome pair 4A has been substituted by chromosome pair 4D (the line contains 2 pairs of 4D chromosomes but no 4A chromosomes). The absence of a fragment in a line (N4AT4D) (for example, in Fig.2 A) indicates that the fragment is located on that nulli chromosome (4A).

and transformed into *E. coli* SURE™ strain (from STRATAGENE, Cat. No. 200294) using the Bio-Rad electrotransformation system according to the manufacturer's instructions. The electroporated cells were plated onto selected medium containing 100 mg/ml carbenicillin. Recombinants were identified using the X-gal and IPTG indicator system. Approximately 300 recombinants were obtained from the chromosome-sorted DNA ligation reaction.

Characterization of the chromosome-specific libraries

Inserts to be used as probes were isolated by EcoRI and XhoI double restriction of recombinant clones. These inserts were labelled and hybridized against Southern blots of a complete set of hexaploid wheat (cv. Chinese Spring) nulli-tetrasomic DNAs digested with EcoRI to determine their chromosomal locations (12). Chinese Spring genomic DNA isolation, enzyme digestion, electrophoresis, Southern blotting, probe labelling and hybridization followed protocols described previously (12).

RESULTS AND DISCUSSION

Recently we have flow karyotyped a wheat (*Triticum aestivum*), TaKB1, cell line (13). Discrete peaks, each arising from chromosomes with similar DNA content were resolved (Fig.1). As in the case of the flow karyotype of tomato cell lines (8), it was unclear whether these peaks arose from several different chromosomes or different chromosomes with similar DNA contents. It was also unclear what the level of contamination was within the peaks due to the fragmentation or clumping of chromosomes. Wheat chromosomes corresponding to a sorted peak (Fig.1) from the cell line, TaKB1, were collected for further analysis.

The cereal genome consists of 80% repetitive sequences (14). Therefore confirmation that a sorted peak corresponds to a specific wheat chromosome requires the identification of single copy sequences in the sorted material which can be mapped to a specific chromosome. In the cereal genome, there is no CpG shortage (15, 16), and therefore many potential recognition sites

Table 1. Chromosomal locations of the HpaII clones from the chromosome-enriched library

Clone	1*	2*	3*	4*	5*	6*	7*	Total
L3-1			3D					
L3-2							7D	
L3-4	1A,1B		3D		5A		7B	
L3-10				4A				
L3-15				4A				
L3-17				4A	5B			
L3-19				4A,4B,4D				
L3-24						6A,6B,6D		
L3-28	1A			4A				
L3-31			3A,3B,3D					
L3-34				4A				
L3-35	1A,1B,1D							
L3-40							7D	
L3-42						6A,6B,6D		
L3-50	1A			4B		6B		
L3-51				4A,4B,4D				
L3-60				4A	5B	6D		
L3-67				4A,4B,4D				
L3-68				4A	5D			
L3-73			3B,3D					
L3-83				4A,4B,4D				
21	4(7)	0(0)	4(7)	12(20)	4(4)	4(8)	3(3)	31(49)

*: Wheat homoeologous group. A: A genome; B: B genome; D: D genome. Numbers in parentheses are the numbers of chromosomal locations.

Table 2. Chromosomal locations of the HpaII clones from the unsorted chromosome library

Clone	1*	2*	3*	4*	5*	6*	7*	Total
HWC5	1B	2B	3A,3B,3D					
HWC7			3B		5B	6B		
HWC9			3A			6D		
HWC11	1B							
HWC23						6A,6B,6D		
HWC24							7A	
HWC33				4B		6B		
HWC34		2B,2D						
HWC45	1B	2D					7A	
9	3(3)	3(4)	3(5)	1(1)	1(1)	4(6)	2(2)	17(22)

*: Wheat homoeologous group. A: A genome; B: B genome; D: D genome. Numbers in parentheses are the numbers of chromosomal locations.

for methylation-sensitive enzymes, although most sites are methylated. Recent studies have indicated that genes/single copy sequences are found in under-methylated or methylation-free islands which may be clustered in the distal regions of cereal chromosomes (17, 18). Unmethylated NotI sites provide landmarks for these islands as do clusters of unmethylated HpaII sites. 80% of short restriction fragments from the wheat genome defined by the methylation-sensitive endonuclease HpaII were single or low copy (19). DNA extracted from flow-sorted chromosomes was therefore digested with HpaII and resultant small HpaII fragments cloned. The DNA inserts from 40 clones picked at random from this HpaII genomic library were screened against Southern blots of digested DNA from nulli-tetrasomic lines of wheat. Twenty-one clones detected low copy sequences which could be mapped to a particular chromosomal location and nineteen clones detected multiple copy sequences which could not be mapped. Examples of the analysis of two clones (L3-15, L3-17) are shown in Fig.2. The analysis indicated that only 50% of the HpaII restriction fragments defined single or low copy sequences on the chromosomes isolated from the cell line.

RFLP analysis of hexaploid wheat showed that single or low copy probes could be categorized into three classes: probes detecting sequences which are chromosome specific (see Fig.2 A); probes detecting sequences on all three homoeologous chromosomes (the same group for each of the A,B,D genomes of wheat); and probes which detected sequences on several different homoeologous chromosome groups (see Fig.2 B). For the purposes of mapping the twenty-one clones which detected low or single copy sequences, the location of the strongest hybridization fragment was taken as being the location of the cloned DNA. In cases where the fragments hybridized with equal intensity, the chromosomal location of these fragments is indicated. If all 40 clones are single or low copy sequences and randomly distributed across the 7 homoeologous groups (21 chromosomes in total), only 2 clones on average would be derived from each chromosome. The twenty-one clones detected sequences which mapped on a total of 49 chromosomal locations, 11 of which were on chromosome 4A (Table 1). None of the unique or low copy number DNA sequences was located on the chromosomes of the homoeologous group 2 and chromosome 7A. In contrast, half of the single or low copy probes detected sequences on 4A. The clones detecting sequences on 4A all had different hybridization patterns on Southern blots of wheat-digested DNA (and barley-digested DNA, in the case of clones also detecting barley sequences) suggesting that these clones are distinct from each other. As a control for the analysis of clones generated from sorted chromosomes, a HpaII genomic library was also constructed from DNA of chromosomes which had not been flow sorted. The sequences detected by 9 clones from this library mapped to 22 chromosomal locations, none of which was on 4A but several DNA fragments were located on the chromosomes of homoeologous group 2 (Table 2).

We conclude from the analysis of 40 random clones, that the HpaII library constructed to the chromosomes collected from one of the peaks was enriched for clones which detected sequences on chromosome 4A. Two of the clones (L3-19, L3-51) have been screened against ditelosomic stocks to determine on which chromosome arm the cloned sequences are located (data not shown). Clone L3-51 detects sequences on the short arm and L3-19 on the long arm of chromosome 4A. Although it is likely that chromosomes in the cell line have rearranged (9), it is clear that some of the resultant chromosomes formed are sufficiently stable to be sorted and homologous enough to the original chromosomes for a useful library to be made. Genetic maps of wheat chromosomes, 4A, 5A and 7B indicate that these chromosomes in modern hexaploid wheat have been subject to a number of translocations. The maps indicate that there are segments on 4AL originating from 5AL and 7BS (20). A cloned sequence derived from chromosome 4A of modern wheat may detect sequences on 5B and/or 5D, if the cloned sequence is derived from the interstitial 5AL segment on this chromosome. L3-17 is an example of such a cloned sequence which maps by RFLP analysis to this translocated region (Xie and Gale, unpublished results). The production of a genomic library enriched for clones detecting sequences on chromosome 4A implies more importantly that a specific wheat chromosome can be flow sorted. These sorted chromosomes therefore provide a source of material for generating chromosome-specific libraries or for use in other techniques currently being developed.

The collection and characterisation of chromosomes by flow sorting the wheat cell line, TaKB1, will yield preparations with various degrees of enrichment of specific chromosomes. The degree of enrichment of probes for a specific chromosome obtained in libraries constructed from the sorted material depends on the source of the material being sorted, quality of the chromosome preparation and the flow cytometer's resolution. However we recently reported a protocol which would help with the enrichment of chromosome specific probes in the libraries generated. Hydroxylapatite-PERT reactions have been used to identify probes mapping in deletions (21), by reannealing euploid DNA with DNA from either nulli-tetrasomic genetic stocks or from stocks containing deletions of individual loci. Approximately 80% of the clones generated by this technique are single or low copy sequences which is similar to the percentage of clones in HpaII libraries constructed from leaf DNA, which are single or low copy sequences. The combination of annealing the DNA from flow-sorted chromosomes (for instance to 4A) in such a reaction with DNA from either nulli-tetrasomic lines (in this instance N4AT4D) or ditelosomic (4AL) lines would further enrich for probes specific for a particular chromosome (4A). We would expect 80% of the clones will be single or low copy sequences and exhibit enrichment for the chromosome targeted. The production of highly chromosome-specific libraries may be possible even though the initial sorted material is not completely pure. The observation that unmethylated sequences are non-randomly distributed along the cereal chromosomes suggests their genes may be relatively clustered (17). The ability to generate large numbers of chromosome-specific probes for these regions containing genes and the availability of a related small genome species may make the concept of chromosome walking in these relatively large plant genomes less daunting.

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