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M. Kubaláková · J. Vrána · J. Číhalíková
H. Šimková · J. Doležel

Flow karyotyping and chromosome sorting in bread wheat (*Triticum aestivum* L.)

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Abstract Previously, we reported on the development of procedures for chromosome analysis and sorting using flow cytometry (flow cytogenetics) in bread wheat. That study indicated the possibility of sorting large quantities of intact chromosomes, and their suitability for analysis at the molecular level. However, due to the lack of sufficient differences in size between individual chromosomes, only chromosome 3B could be sorted into a high-purity fraction. The present study aimed to identify wheat stocks that could be used to sort other chromosomes. An analysis of 58 varieties and landraces demonstrated a remarkable reproducibility and sensitivity of flow cytometry for the detection of numerical and structural chromosome changes. Changes in flow karyotype, diagnostic for the presence of the 1BL·1RS translocation, have been found and lines from which translocation chromosomes 5BL·7BL and 4AL·4AS-5BL could be sorted have been identified. Furthermore, wheat lines have been identified which can be used for sorting chromosomes 4B, 4D, 5D and 6D. The ability to sort any single arm of the hexaploid wheat karyotype, either in the form of a ditelosome or a isochromosome, has also been demonstrated. Thus, although originally considered recalcitrant, wheat seems to be suitable for the development of flow cytogenetics and the technology can be applied to the physical mapping of DNA sequences, the targeted isolation of molecular makers and the construction of chromosome- and arm-specific DNA libraries. These approaches should facilitate the analysis of the complex genome of hexaploid bread wheat.

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M. Kubaláková · J. Vrána · J. Číhalíková · H. Šimková
J. Doležel (✉)
Laboratory of Molecular Cytogenetics and Cytometry,
Institute of Experimental Botany, Sokolovská 6,
CZ-77200 Olomouc, Czech Republic
e-mail: dolezel@ueb.cas.cz
Tel.: +420-68-5228521, Fax: +420-68-5228523

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Introduction

Genome mapping in plants is advancing rapidly. As a result of an international collaborative effort, the genome sequence of the first flowering plant, *Arabidospis thaliana* (1C, approximately 125 Mbp), was announced in December 2000 (Arabidopsis Genome Initiative 2000). The sequence is almost complete but excludes some telomeric and centromeric regions. The information obtained, as well as the problems encountered during the *Arabidopsis* sequencing project, are of great value for analysing genomes of economically important crops. With some exceptions like rice (*Oryza sativa*), genomes of crop-plant species are many times lager. Hexaploid bread wheat (*Triticum aestivum*) with a genome size of about 17,000 Mb (1C = 17.32 pg DNA, Bennett and Smith 1976), i.e. 130-times larger than that of *Arabidopsis*, is an extreme example. In addition to being complex, the genome consists of three related (homoeologous) genomes (A, B and D). These characters make the genome of bread wheat difficult to analyse.

The analysis of the human genome (about six-times smaller than that of bread wheat) has been facilitated by the availability of subgenomic, chromosome-specific, DNA libraries. The libraries were constructed using chromosome fractions purified by flow cytometry (Van Dilla et al. 1990; McCormick et al. 1993; Nizetic et al. 1994), and were especially important for mapping centromeric and telomeric repeat-containing regions and sequences showing homology with other human chromosomes (Dunham et al. 1999; Hattori et al. 2000). Procedures for flow cytometric analysis and the sorting of mitotic chromosomes (flow cytogenetics) have also been developed for animal species (Dixon et al. 1992; Ferguson-Smith 1997) where flow-sorted chromosomes have been used for the construction of chromosome-specific DNA libraries (Miller et al. 1992; Balajee et al.

1995), the generation of chromosome-specific painting probes (Burkin et al. 1997; Yang et al. 1999), and for targeted isolation of molecular markers (Shepel et al. 1998; Lan et al. 1999).

Development of flow cytogenetics in plants has been delayed due to problems with the preparation of suspensions of intact chromosomes and difficulties in discriminating single chromosome types (Doležel et al. 1994). These problems have been overcome during the last decade and flow cytogenetics has been developed for a range of plant species (Doležel et al. 1999, 2001). These included model plants like *Silene latifolia*, which is used to study sex determination and the evolution of sex chromosomes (Veuskens et al. 1995; Kejnovský et al. 2001). Economically important crops included legumes and cereals. In legumes, flow cytogenetics has been developed for *Vicia faba* (Doležel et al. 1992), *Pisum sativum* (Gualberti et al. 1996), and *Cicer arietinum* (Rychtarová et al., submitted). Examples of the cereals where flow cytogenetics has been developed include *T. aestivum* (Wang et al. 1992; Lee et al. 1997; Vrána et al. 2000), *Hordeum vulgare* (Lysák et al. 1999) and *Zea mays* (Lee et al. 1996).

Lee et al. (1997) succeeded in generating reproducible flow karyotypes after analysing isolated wheat chromosomes by flow cytometry. However, they were not able to sort single chromosomes. Following this, they used a ditelosomic wheat line to sort a few hundred 1DS chromosome arms (Gill et al. 1999). Our previous results indicated the possibility to obtain high-resolution flow karyotypes in bread wheat and to sort a large quantity of mitotic 3B chromosomes at high purity (Vrána et al. 2000). DNA obtained from the sorted chromosomes was of high-molecular-weight and suitable for PCR and the construction of large-insert DNA libraries. The availability of such libraries would greatly simplify the analysis of the complex wheat genome. However, broader application of flow cytogenetics in wheat was hampered by the inability to sort other chromosome types. The aim of this study was to explore the possibility to sort other chromosome types and single chromosome arms in wheat. The results obtained after the screening of 58 lines showed that other wild-type chromosomes, translocation chromosomes and chromosome arms could be sorted at high purity. These results will form the basis for broader use of flow cytogenetics in wheat genome mapping.

Materials and methods

Plant material

Seeds of wheat (*T. aestivum* L., 2n = 6*x* = 42) "Chinese Spring" with a standard karyotype, "Chinese Spring" carrying di-iso5BL, and "Cappelle Desprez" carrying translocation chromosome 5BL·7BL were kindly provided by Dr. R. Koebner (John Innes Centre, Norwich, UK). Seeds of "Chinese Spring" carrying translocation chromosome 4AL·4AS-5BL, "Chinese Spring" carrying di-iso5BL and "Pavon" ditelosomic 1BS were kindly provided by Professor A. J. Lukaszewski (University of California, Riverside, USA). Variety "Emma" with a standard karyotype and "Emma" with a 1RS·1BL translocation were provided by Dr. M. Molnár-Láng (Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary). Seeds of other wheat cultivars were obtained from Dr. Z. Stehno (Research Institute of Crop Production, Prague, Czech Republic) and Dr. Z. Kryštof (Agricultural Research Institute, Kroměříž, Czech Republic). Dihaploid wheat line M18 was obtained from Dr. L. Kučera (Research Institute of Crop Production, Prague, Czech Republic).

Preparation of chromosome suspensions

The preparation of chromosome suspensions from synchronized root tip meristems was performed as described by Vrána et al. (2000). All seeds were germinated in the dark at 25 ± 0.5 °C on moistened filter paper in glass Petri dish for 3 days to achieve optimal root length (2–3 cm). Root tip cells were synchronised by incubation in 2 mM hydroxyurea for 18 h, then the seedlings were transferred for 4.5 h to Hoagland's solution for the recovery from the hydroxyurea block and accumulated in metaphase by a treatment with amiprophos-methyl 2.5 mM for 2 h and overnight treatment in ice water (1–2 °C). Synchronised root tips were fixed at 5 °C for 20 min in 2% (v/v) formaldehyde in Tris buffer (Doležel et al. 1992). After three washes in Tris buffer, root tips were cut 1 mm from the tip and homogenised at 20,000 rpm for 10 s in 1 ml of LB01 lysis buffer (Doležel et al. 1989) using a Polytron PT1300D homogeniser (Kinematica AG, Littau, Switzerland). The crude suspension was passed through a 50-µm pore-size nylon mesh to remove large tissue fragments.

Flow cytometry

Chromosome analysis and sorting was performed on the FACSVantage flow cytometer (Becton Dickinson, San José, USA) equipped with an argon ion laser set to multiline UV and 300 mW output power. A solution of 50 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 analysed at rates of 200–400 particles per s. DAPI fluorescence was measured through a 424/44 band-pass filter in front of a fluorescence 1 (FL1) detector. Relative fluorescence intensities, which corresponded to the relative DNA content of gated populations, were acquired on histograms of the FL1 pulse area. Approximately 10,000 chromosomes were analysed in each sample. For chromosome sorting, gates were set on a dot plot of the FL1 pulse area versus FL1 pulse width and chromosomes were sorted at rates of 5–10 per s.

Two-step sorting (Lucretti et al. 1993) was used to achieve higher purity in fractions of long chromosomes. At least 50,000 chromosomes were sorted into a polystyrene tube containing 250 µl of LB01. The fraction enriched for given chromosome was stained by DAPI (2 µg/ml) and used for sorting as described above.

Cycling-primed in situ labelling (C-PRINS)

The identity and purity of sorted fractions was determined after labelling of GAA microsatellites (Kubaláková et al. 2000). Briefly, 2,000 chromosomes were sorted onto a microscope slide into a 15-µl drop of PRINS buffer supplemented with 5% sucrose. After air-drying, 25 µl of EasiSeal frame (Hybaid, Surrey, UK) was fixed to the slide over the specimen area and 20 µl of PRINS reaction mix were 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, 1.5 U/25 µl Dynazyme II DNA polymerase (Finnzymes OY, Finland), and 4 µM $MgCl_2$ in 1 × PCR buffer. $(GAA)_7$ and $(CTT)_7$ primers, which amplify GAA microsatellites (Kubaláková et al. 2000), or AS-A and AS-B primers, which amplify part of the Afa repeat (Nagaki et al. 1998), were used. PRINS was performed on a PTC100 thermal cycler (MJ Re-

search, Watertown, USA) equipped with an actively controlled heated plate. The reaction consisted of the following steps: 20 cycles of 45 s at 91 °C, 45 s at 55 °C, and 45 s at 72 °C. The extension was prolonged to 5 min in the final cycle. The PRINS reaction was stopped in a stop buffer (0.5 M NaCl, 0.05 M Na_2EDTA, pH 8.0) for 5 min at 70 °C, 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, USA).

Fluorescence microscopy

The preparations were evaluated using an Olympus BX 60 microscope equipped with filter sets appropriate for fluorescein and PI fluorescence. The images of fluorescein and PI fluorescence were acquired separately with a b/w CCD camera, which was interfaced to a PC running the ISIS software (Metasystems, Altlussheim, Germany). The images were superimposed after contrast and background optimisation, inverted to a grey scale and assembled to individual plates using Adobe Photoshop software (Adobe Systems, San José, USA).

Polymerase chain reaction

As the identity of flow-sorted D chromosomes could not be unequivocally determined after GAA banding, PCR with chromosome-specific primers was used; 1,000 chromosomes of each type were sorted into 0.5-ml PCR tubes containing 20 µl of sterile deionised water. The tubes with sorted chromosomes were frozen and kept at –20 °C. Before the reaction, the chromosomes were thawed and PCR premix was added to reach the reaction volume of 50 µl. Final concentrations of the reagents were: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM dNTPs, 2.5 U/50 µl *Taq* DNA polymerase (Sigma, St. Louis, USA). Primers for chromosome-specific microsatellite markers (Röder et al. 1998) WMS190 for 5D and WMS194 for 4D, were used at 0.5 µM concentration. PCR was performed under the following conditions: denaturation step 4 min at 94 °C followed by 40 cycles consisting of 1 min denaturation at 92 °C, 1 min annealing at 47 °C for WMS194 and 57 °C for WMS190, 2 min extension at 72 °C, and final extension at 72 °C for 10 min. PCR products were analysed by electrophoresis on a 1.5% agarose gel.

Results

The analysis of chromosomes isolated from "Chinese Spring" resulted in flow karyotypes consisting of three composite peaks (labelled I, II and III) corresponding to various chromosome types (Vrána et al. 2000) and a peak corresponding to chromosome 3B (Fig. 1a). Chromosome 3B could be sorted at 89% purity as determined by microscopic evaluation of chromosomes after labelling with GAA microsatellites (Fig. 1a, insert). However, the purity could be increased to 95% after two-step sorting. Similar flow karyotypes were obtained in 33 other genotypes where chromosome 3B could be sorted (Table 1). Besides modern varieties, this set included ten Czech landraces.

In addition to chromosome 3B, other chromosome types could be sorted from lines carrying chromosomes with their length changed due to translocations. Thus, chromosome 5BL·7BL, which is considerably longer then the remaining chromosomes could be easily discriminated on the flow karyotype of "Cappelle Desprez" (Fig. 1b). After two-step sorting, the purity in sorted 5BL·7BL fractions exceeded 97%. In addition to translo-

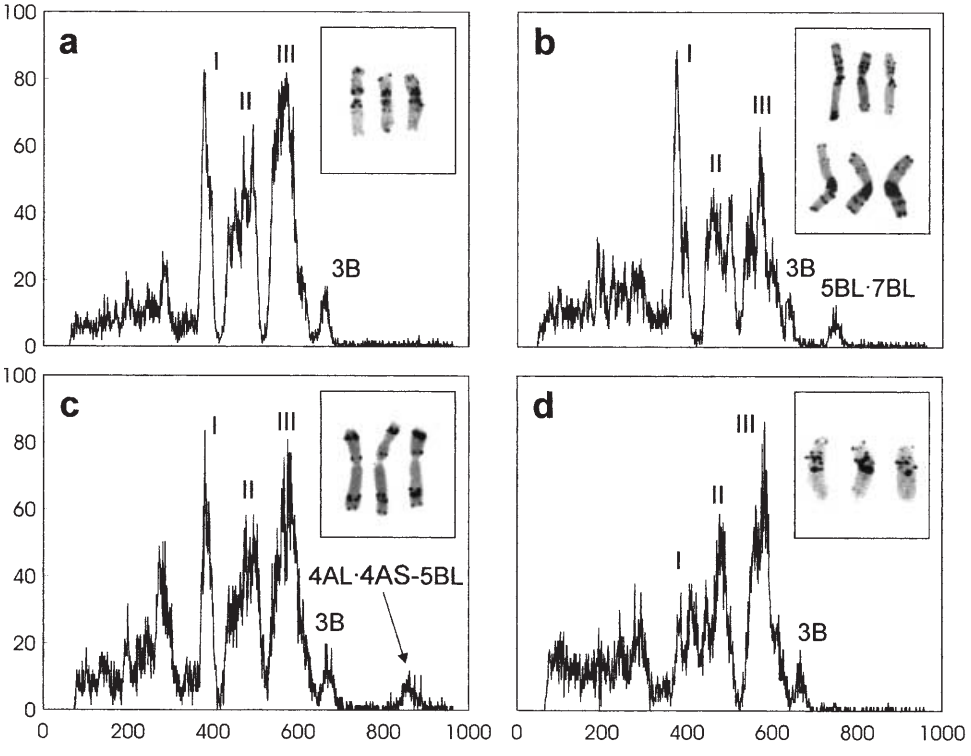

Fig. 1a–d Flow karyotypes obtained after analysis of DAPI-stained chromosome suspensions prepared from four wheat lines. **a** The flow karyotype of "Chinese Spring" consists of three composite peaks (*I–III*) representing groups of chromosomes and the peak representing chromosome 3B (*insert*: chromosome 3B after GAA banding); **b** the flow karyotype of "Cappelle Desprez" with three composite peaks (*I–III*) and well-discriminated peaks representing 3B and 5BL·7BL [*insert*: chromosomes 3B (*top*) and 5BL·7BL (*bottom*) after GAA banding]; **c** the flow karyotype of "Jubilar" with three composite peaks (*I–III*) and peaks representing 3B and 4AL·4AS-5BL (*insert*: GAA-banded 4AL·4AS-5BL); **d** the flow karyotype of "Zlatka" characterised by overlapping composite peaks *I and II*, composite peak *III* and the peak representing 3B (*insert*: GAA-banded 3B). X axis: relative DAPI fluorescence intensity; Y axis: number of events

Table 1 List of wheat varieties used in this study and the chromosome types that could be sorted

Variety	Source[a]	Chromosome translocation	Sortable chromosomes
Amigo	3	1RS·1AL, 1BL·1BS-3Ae1L	5D, 3B
Agra	2	1RS·1BL	
Athlet	6	1RS·1BL	5BL·7BL, 5A + 4B
Aurora	6	1RS·1BL	
Avalon	2		3B
Bernina	6	1RS·1BL	
Bezostaja 4	6		3B
Bila od Dukovan[b]	6		3B
Boka	6		3B
Boleslavská bělka[b]	6		3B
Boleslavská vouska[b]	6		3B
Cappelle Desprez	1	5BL·7BL, 3B·3D	3B·3D, 5BL·7BL
Chinese Spring	1, 4		3B
Chinese Spring	4	4AL·4AS-5BL	4AL·4AS-5BL, 3B
Chinese Spring	1, 4	diiso5BL	5BL·5BL, 3B
Chinese Spring	4	ditelo1BS	1BS, 3B
Chlumecká 12[b]	6		3B
Dětenická červená vouska[b]	6		3B
Drake	1	1RS·1BL	5BL·7BL
Emma	5		3B
Emma with 1RS	5	1RS·1BL	
Famulus	6		3B, 5BL·7BL, 5A + 4B, 6B
Florida	3	1RS·1BL	5BL·7BL
Hareward	6	1RS·1BL	
Hornet	2	1RS·1BL	5BL·7BL
Iljičovka	6		3B
Iris	2, 6	1RS·1BL	
Jubilar	6	5BL·7BL	3B, 5BL·7BL
Kavkaz	6	1RS·1BL	
Kormoran	6	1RS·1BL	
Livia	6	1RS·1BL	
M18	3	1RS·1BL	5BL·7BL
Maja	2		3B
Mironovskaja 808	6		3B
Mona	2	1RS·1BL	4D, 1D + 6D, 3B
Norman	2		3B, 5BL·7BL
Pantus	6	1RS·1BL	4B
Pavon	4	ditelo1BS	1BS, 3B
Přesívka Červený Oujezd[b]	6		3B
Rektor	2		3B
Rexia	6		3B
Rokycanská sametka[b]	6		3B
Sadomierka[b]	6		3B, 4D, 1D + 6D
Saxana	6		3B
Sensor	6	1RS·1BL	5BL·7BL
Senta	2	1RS·1BL	5A + 4B + 5B
Sida	2	1RS·1BL	4B
Simona	6	1RS·1BL	5BL·7BL
Slovenská B[b]	6		3B, 4D, 1D + 6D
Sofia	6	1RS·1BL	3B
Sparta	2	1RS·1BL	3B
Sperber	2		3B
Titus	6	1RS·1BL	3B
Tonic	2		3B
Veery "S"	6	1RS·1BL	3B
Vlada	3		3B
Zlatka	6	5A·6D, 3D·5D	3B
Židlochovická jubilejní osinatá[b]	6		3B

[a] The seeds were kindly provided by the following researchers:
1. R. Koebner, John Innes Centre, Norwich, UK
2. Z. Kryštof, Agricultural Research Institute, Kroměříž, Czech Republic
3. L. Kučera, Research Institute of Crop Production, Prague, Czech Republic
4. A. Lukaszewski, University of California, Riverside, USA
5. M. Molnár-Láng, Agricultural Research Institute, Martonvásár, Hungary
6. Z. Stehno, Research Institute of Crop Production, Prague, Czech Republic

[b] Czech landrace

cation 5B·7B, the variety carries a translocation 3B/3D (Riley 1967), which results in chromosome 3B being shorter by about 3% as compared to the 3B of "Chinese Spring". This alteration is reflected by a change in the ratio of peak position 3B versus composite peak I from 1.73 in "Chinese Spring" to 1.68 in "Cappelle Desprez". As a result of structural chromosome changes, the profiles of the three composite peaks (I, II, III) on the flow karyotype differed from those of "Chinese Spring". Similar results were obtained in a "Chinese Spring" line carrying chromosome 4AL·5BL and in "Jubilar" (Fig. 1c). On the other hand, no other chromosome than 3B could be sorted in "Zlatka", which carries translocations between chromosomes 5A and 6D, and 3D and 5D (Košner

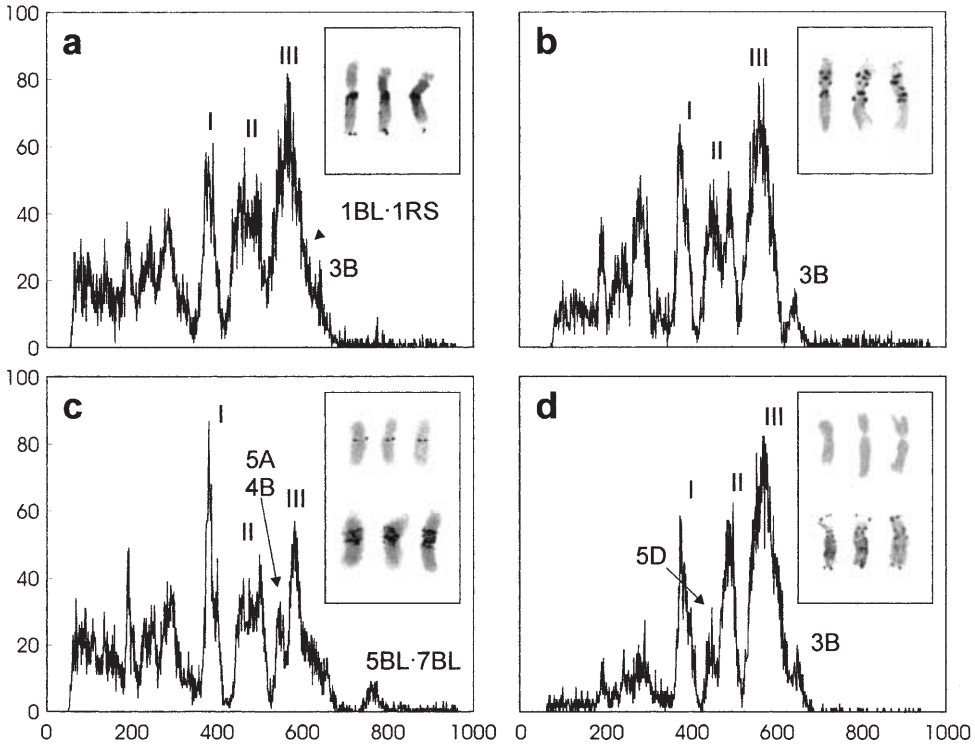

Fig. 2a–d Flow karyotypes obtained after analysis of DAPI-stained chromosome suspensions prepared from four wheat lines. **a** The flow karyotype of "Emma" carrying the 1BL·1RS translocation consists of three composite peaks (*I–III*) with the peaks of 1BL·1RS and 3B overlapping (*insert*: chromosome 1BL·1RS after GAA banding, note the presence of a secondary constriction); **b** the flow karyotype of "Emma" without 1BL·1RS showing a clear discrimination of the peak representing 3B (*insert*: GAA-banded 3B); **c** the flow karyotype of "Athlet" with three composite peaks (*I–III*) and the peak representing 5BL·7BL. Note that the peak representing chromosomes 5A and 4B is separated from composite peak III (*insert*: GAA-banded 5A and 4B); **d** the flow karyotype of "Amigo" with three composite peaks (*I–III*) and the peak representing 3B. Note that the peak representing chromosome 5D is separated from composite peak II (*insert*: 5D displaying no GAA bands and 5D after Afa banding). X axis: relative DAPI fluorescence intensity; Y axis: number of events

and Bareš 1979). However, due to the translocations, peak I is almost absent and the flow karyotype is characterised by a shoulder between composite peaks I and II (Fig. 1d).

A number of wheat lines carry the short arm of chromosome 1 of rye (1RS) in the form of a whole-arm chromosome translocation replacing the short arm of wheat group-1 chromosomes. When inserted into wheat, it significantly improves variety performance (Schlegel 1997; Graybosch 1998; Berzonsky and Francki 1999). We have analysed a set of 25 lines aimed to sort recombinant chromosome 1BL·1RS (Table 1) and found that the peak of 1BL·1RS was located between peak III and the peak of chromosome 3B, resulting in a composite peak involving chromosomes of peak III, chromosome 1BL·1RS, and chromosome 3B (Fig. 2a). This type of flow karyotype was characteristic for nearly all 1BL·1RS lines (compare Fig. 2a and b). The location of 1BL·1RS on flow karyotypes was confirmed by sorting. Sorted-chromosome 1BL·1RS was identified based on the GAA banding pattern of the long arm and the presence of a secondary constriction on its short arm (Fig. 2a, insert). Due to peak overlap, chromosome 1BL·1RS could be sorted only at 60% purity. Flow karyotyping of lines which were reported to carry 1BL·1RS, showed that some of them ("Athlet", "Drake", "Famulus", "Florida", "Hornet", "Senzor", "Simona") and a dihaploid line M18 contained a well-separated peak to the right of peak 3B. Flow sorting demonstrated that the peak corresponded to recombinant chromosome 5BL·7BL (Figs. 2c and 3).

Variety "Amigo" was selected as representative of genotypes with 1RS translocated to the long arm of 1A. In addition to translocated-chromosome 1AL·1RS, the variety carries chromosome 1BL·1BS-3Ae#1L, which has a segment from chromosome 3Ae#1 of *Agropyron elongatum* (Jiang et al. 1994). None of the two chromosomes could be individually sorted. As 1AL·1RS is shorter compared to 1RS·1BL, it did not form a shoulder on the right side of composite peak III but almost completely overlapped with the peak resulting in a broad composite peak III (Fig. 2d). The presence of 1AL·1RS in composite peak III was confirmed after flow sorting based on the GAA banding pattern and the presence of a secondary constriction on its short arm (Fig. 4). Due to the translocation involving chromosome 1A, which is normally located in composite peak II (Vrána et al. 2000), the peak corresponding to chromosome 5D was resolved from peak II and the chromosome could be sorted at purities exceeding 97%. As the chromosome exhibits no GAA bands, its identity was confirmed after

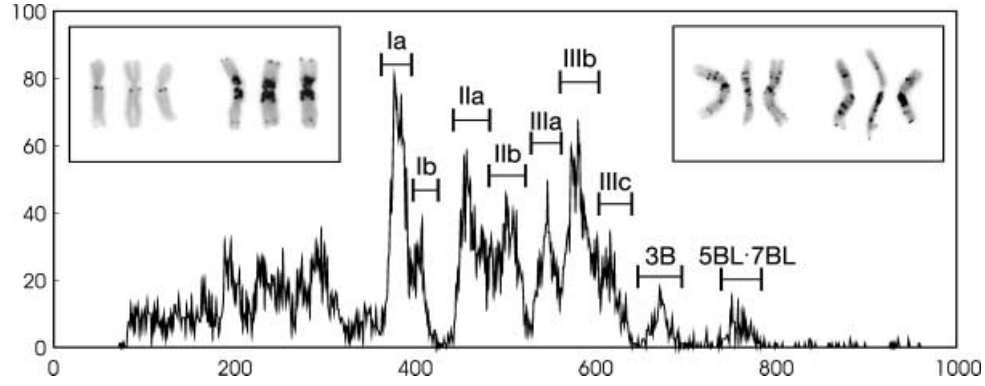

Fig. 3 Flow karyotype of "Famulus" obtained after analysis of DAPI-stained chromosome suspension. Each of the composite peaks *I–III* is resolved into two or three more-or-less discriminated peaks (labelled *a–c*). In addition, peaks representing chromosomes 3B and 5BL·7BL are clearly discriminated. *Left insert*: chromosomes 5A and 4B; *right insert*: 3B and 5BL7BL. The chromosomes were identified after GAA banding. X axis: relative DAPI fluorescence intensity; Y axis: number of events

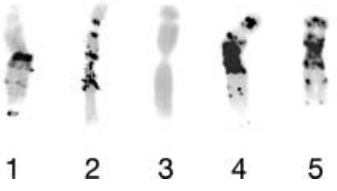

Fig. 4 Flow-sorted chromosomes with visible secondary constriction after DAPI staining. Key: *1* = 1RS·1BL of "Kavkaz", *2* = 6B of "Kavkaz", *3* = 1RS·1AL of "Amigo", *4* = 1BL·BS-3Aeč 1L of "Amigo", *5* = 6B of "Amigo". The chromosomes were identified after GAA banding

fluorescent labelling of Afa repeats (Nagaki et al. 1998, Fig. 2d, insert) and by PCR with chromosome-specific primers (data not shown).

An unexpected possibility to sort chromosome 5D from "Amigo" stimulated the flow karyotyping of other lines for separated peaks corresponding to wild-type chromosomes. While flow karyotypes in most of lines exhibited composite peaks I, II and III with profiles similar to those of "Chinese Spring", several exceptions were identified. Thus, a small peak was detected to the left of composite peak III on the flow karyotype of "Athlet" (Fig. 2c). The peak represented chromosomes 5A and 4B (Fig. 2c, insert), which could be sorted simultaneously. It should be noted that in all flow karyotypes, the frequency of events in individual peaks corresponded fairly well with the actual number of chromosome types identified after sorting. This is demonstrated on the flow karyotype of "Famulus" (Fig. 3, Table 2). Due to this, it was possible to predict the number of chromosome types that could be sorted from each peak, and to identify peaks corresponding to single chromosome types.

The flow karyotypes of "Pantus" and "Sida" exhibited a pattern typical for 1BL·1RS lines with the 3B peak overlapping the composite peak III (Fig. 5a). However, extra peak to the right of peak III was detected. It was found to represent chromosome 4B, which could be sorted in 94% purity. This chromosome was longer compared to the 4B of "Chinese Spring" due to an extra terminal segment on its long arm. As microscopic analysis revealed no GAA banding of the terminal segment on 4BL (Fig. 5a, insert), we were not able to determine whether it was a duplication or translocation. In "Mona", "Rexia", and to a lesser extent also in some other genotypes, the composite peak I was split into two smaller peaks, one of them suspected to represent only one chromosome type (Fig. 5b). It was found that the peak on the left corresponded to chromosomes 1D and 6D, while the smaller peak represented chromosome 4D. Identification of chromosome 4D was confirmed by PCR. When analysing the origin of this type of flow karyotype, it was found that the composite peak I has also been clearly split in "Iljičovka", a parental variety of "Mona" and "Rexia", and in some other Russian varieties (e.g. "Kavkaz"). Interestingly, the same pattern was found also in some Czech landraces e.g. "Slovenská B" and "Sadomierka".

As flow sorting of defined parts of chromosomes would enable more targeted genome analysis and mapping, the last series of experiments were focused on the sorting of single chromosome arms. According to theoretical considerations, the use of telosomic lines would permit the sorting of all short arms and most of the long arms (Table 3). The remaining long arms, 3BL and 5BL, which would overlap with peaks representing other chromosomes, were expected to be sortable as isochromosomes. This hypothesis was tested in two wheat lines. The analysis of "Chinese Spring" line carrying iso5BL showed that the chromosome could be easily discriminated, and purities exceeding 85% could be achieved after two-step sorting (Fig. 5c). The flow karyotype of "Pavon" ditelosomic for the short arm of 1B (dt1BS) contained a distinct peak representing 1BS (Fig. 5d) and the chromosome could be sorted at 82% purity.

Labelling of GAA microsatellites resulted in a chromosome banding pattern similar to C-banding (Friebe and Gill 1994). The banding pattern exhibited polymorphism, the most extensive being in chromosome 3B (Fig. 6a). While all marker bands (S1.3, S1.5, S2.1 a L1.3, L2.1) could be observed on this chromosome, the polymorphism mainly concerned band size, with the

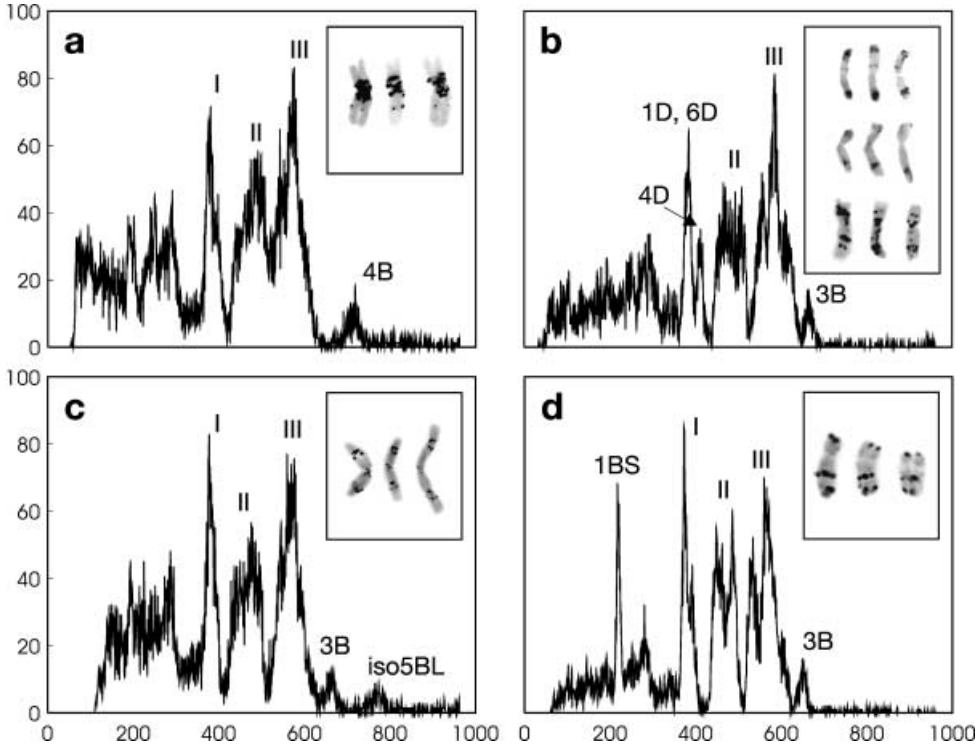

Fig. 5a–d Flow karyotypes obtained after analysis of DAPI-stained chromosome suspensions prepared from four wheat lines. **a** The flow karyotype of "Panthus" consists of three composite peaks (*I–III*) and peak representing chromosome 4B (insert: chromosome 4B after GAA banding); **b** the flow karyotype of "Mona" with composite peak I split into two peaks, the first representing chromosomes 1D and 6D and the second representing chromosome 4D. In addition, the peak of 3B is clearly discriminated (*insert*: GAA-banded 1D, 6D and 4D); **c** the flow karyotype of "Chinese Spring" carrying iso5BL. In addition to the three composite peaks (*I–III*) and peak of 3B, peak representing iso5BL is clearly discriminated (*insert*: GAA-banded iso5BL); **d** the flow karyotype of "Pavon" carrying 1BS shows three composite peaks (*I–III*), peak of 3B and the clearly discriminated peak of 1BS (*insert*: GAA-banded 1BS). X axis: relative DAPI fluorescence intensity; Y axis: number of events

Table 2 Frequency of events and chromosome types that were sorted from individual peaks of the flow karyotype of "Famulus"

Item	Peak									Total
	Ia	Ib	IIa	IIb	IIIa	IIIb	IIIc	3B	5BL·7BL	
Number of events (per cent of total)	1,132 (18.0)	421 (6.6)	970 (15.3)	999 (15.8)	632 (10.0)	1,338 (21.1)	445 (7.0)	210 (3.3)	184(2.9)	6,331(100)
Number of chromosomes[a]	3.75	1.40	3.22	3.31	2.10	4.44	1.48	0.70	0.61	21
Number of sorted chromosomes	3	1	3	3	2	5	2	1	1	21
Sorted chromosome types[b]	5BS·7BS 1D 4D	6D	1A 3D 5D	2D 6A 7D	5A 4B	2A 3A 4A 6B 7A	1B 2B	3B	5BL·7BL	

[a] Predicted based on the frequency of events in individual peaks. Note that this estimation is compromised by the presence of debris background and by preferential loss of longer chromosomes during chromosome isolation

[b] Chromosome types prevailing in the sorted fraction. Due to peak overlaps, sorted fractions were occasionally contaminated with other chromosome types. Chromosomes that could be sorted at high purity are printed in bold

most-polymorphic bands being S1.3., L2.3 and L2.4. Similar 3B banding patterns were found both in modern lines and old landraces. For instance, the same banding pattern was observed in "Rokycanka", a Czech landrace, and in modern varieties "Jubilar" and "Famulus". Smaller variation of the GAA banding pattern was observed in 5BL.7BL. Some varieties differed by the presence of a small subtelomeric band (Fig. 6b). The analysis of GAA banding pattern also discovered striking intravarietal variation; for example, we found that in "Boleslavská

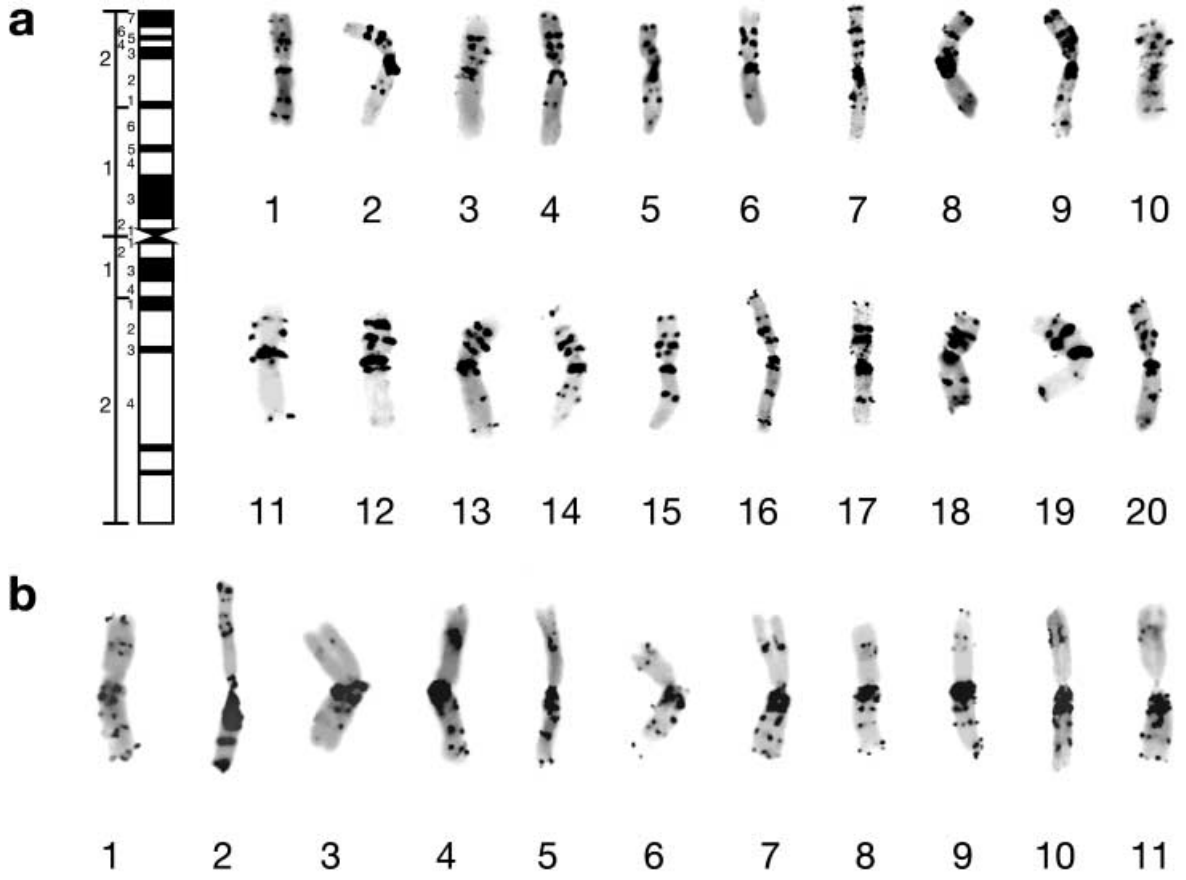

Fig. 6a, b Polymorphism in GAA banding of wheat chromosomes. **a** Chromosome 3B. Idiogram of banded chromosome 3B was taken from Gill et al. (1991), modified. Key: *1* = "Amigo", *2* = "Avalon", *3* = "Athlet", *4* = "Bila od Dukovan", *5* = "Boleslavská bielka", *6* = "Dětenicka červená vouska", *7* = "Drake", *8* = "Florida", *9* = "Jubilar", *10* = "Kavkaz", *11* = "Přesívka Červený Oujezd", *12* = "Rektor", *13* = "Rokycanská sametka", *14* = "Sadomierka", *15* = "Saxana", *16* = "Slovenská B", *17* = "Sparta", *18* = "Titus", *19* = "Tonic", *20* = ''Židlochovická jubilejní osinatá''. **b** Chromosome 5BL·7BL. Key: *1* = "Athlet", *2* = "Cappelle Desprez", *3* = "Drake", *4* = "Famulus", *5* = "Florida", *6* = "Hornet", *7* = "Jubilar", *8* = "M18", *9* = "Norman", *10* = "Senzor", *11* = "Simona"

Table 3 Predicted peak positions of telosomes and isochromosomes on the flow karyotype of "Chinese Spring"[a]

Type	A							B							D						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Short arm																					
Telosome	**191**	**272**	**250**	**220**	**205**	**233**	**283**	**219**	**293**	**300**	**271**	**202**	**289**	**250**	**156**	**219**	**222**	**160**	**179**	**225**	**241**
Isochromosome	383	544	500	**441**	411	467	565	**437**	587	600	**543**	404	578	500	**311**	**439**	446	**322**	**359**	450	481
Long arm																					
Telosome	**364**	**353**	**325**	**375**	**370**	**257**	**283**	**372**	**352**	390	**299**	403	**346**	**375**	**264**	**285**	**312**	**289**	**341**	**270**	**265**
Isochromosome	**727**	707	**650**	**750**	**740**	514	565	**743**	704	**780**	597	**807**	693	**750**	529	571	624	579	682	**540**	529

[a] Peak positions were calculated based on the arm length given by Gill (1987) and by considering 1 μm as equivalent to the relative DNA content of 50 channels. Peak positions of sortable chromosomes are printed in bold

bělka" 10% of 3B chromosomes possess an additional band on the long arm.

Discussion

The results obtained after flow karyotyping of 58 wheat lines demonstrated remarkable reproducibility and sensitivity of the method for the detection of numerical and structural chromosome changes. An agreement between the number of events in each peak on the flow karyotype and the number of chromosome types represented by that peak proved the suitability of flow karyotyping for the detection of numerical chromosome changes. Furthermore, the position of peaks on flow karyotypes agreed well with the relative chromosome size. This will permit

the planning of chromosome sorting experiments in the future and may also be used to predict the possibility to detect structural chromosomal changes.

The presence of the whole arm 1BL·1RS translocation resulted in the fusion of composite peak III and the peak of chromosome 3B, and the modified flow karyotype was diagnostic for the presence of 1BL·1RS. An interesting finding was the observation of a secondary constriction on the short arm of 1BL·1RS in all lines carrying this translocation. A secondary constriction was also observed on the short arm of 1AL·1RS (Fig. 4). These observations contrast with several reports (Mettin et al. 1978; Merker 1982; Cermeno et al. 1984) where the presence of secondary constriction and/or an active nucleolus organizing region was not detected on 1RS after acetocarmine, Feulgen or silver staining. The presence of only one or two pairs of satellite chromosomes (1B and 6B) has been considered diagnostic for the 1RS translocation (Berzonsky and Francki 1998). The reason for this discrepancy may be the weak staining of GC-rich rDNA repeats by DAPI, which we used to stain chromosomes.

Flow karyotyping was sensitive enough to detect the presence of other chromosome translocations, including 5BL·7BL, which was also present is several 1BL·1RS lines (Table 1). To our knowledge, the presence of this chromosome in some of the lines ("Athlet", "Drake", "Famulus", "Florida", "Hornet", "Senzor", "Simone") has not been reported previously. The ability to detect a translocation chromosome was further expanded to follow its transmission. For example, our results indicate that chromosome 5BL·7BL present in "Senzor" originated from "Florida". Even if a single translocation chromosome could not be detected other features of the flow karyotype, e.g. the split composite peak I, most probably reflecting differences in chromosome length, were diagnostic and their transmission could be followed. This was clearly demonstrated in case of "Mona" and "Rexia" which inherited this character from a Russian parental variety "Iljičovka". The reason why some Czech landraces ("Slovenská B", "Sadomierka") share the same character is not clear. However, one cannot exclude the possibility that they were included in the Russian breeding programme.

In many cases, minor differences in flow karyotypes were detected. This could be due to the presence of still undetected translocations and/or due to chromosome polymorphism. Our observation of several chromosome types detected significant variation in GAA banding. While the differences in C banding among wheat lines have already been described (Friebe and Gill 1994), we have detected significant intravarietal polymorphism. The origin of this type of variation is not clear, and may be due to the multiline nature of some of the wheat varieties and/or due to seed contamination. Nevertheless, our results clearly show the advantage of analysing large numbers of isolated chromosomes in contrast to classical analyses of limited numbers of metaphase plates.

One of the most important results of the study is a demonstration of the possibility to sort several types of chromosomes, in addition to 3B as reported originally (Vrána et al. 2000). Thus, in addition to translocation chromosomes 5BL·7BL and 4AL·4AS-5BL, it has been possible to obtain purified fractions of chromosomes 4B, 4D, 5D and 6D. While peaks of chromosome 4D ("Mona") and 5D ("Amigo") were exposed due to structural changes of other chromosomes, chromosome 4B formed a separate peak in "Panthus" and "Sida" most probably due to its increased length (DNA content) resulting from a translocation and/or duplication. In several cases, two chromosome types could be sorted simultaneously (5A + 4B, 1D + 6D). Although less useful then single chromosome fractions, the ability to obtain subgenomic fractions in wheat might be useful for some studies.

This work expands the results of Gill et al. (1991) who reported the sorting of 571 1DS chromosome arms from two separate experiments. Our procedure enables the sorting of at least a 50-times higher number of chromosome arms from the same number of root tips. Furthermore, the use of GAA banding permits the unambiguous identification of sorted arms and hence the precise estimation of purity in the sorted fractions. The use of isochromosomes represents a novel approach, which permits obtaining a higher quantity of chromosome arm-specific DNA. As there is no overlap with peaks representing chromatids and chromosome debris, the purity in isochromosome fractions is higher compared to fractions of chromosome arms. Unfortunately, this approach is not feasible for all chromosome arms due to overlap with peaks representing chromosomes (Table 3). Together with the results of Gill et al. (1991), the present study indicates that the rational choice of telosomic lines and lines carrying isochromosomes should permit sorting of most of the 42 chromosome arms of hexaploid wheat.

To summarise, this study showed a surprising sensitivity of flow karyotyping in the detection of various numerical and structural chromosome changes. Following our previous study (Vrána et al. 2000), this work considerably expands the number of chromosomes that can be sorted at high purity. Furthermore, it demonstrates successful sorting of single chromosome arms of hexaploid wheat. Thus, although originally considered recalcitrant, wheat seems to be suitable for the development of flow cytogenetics. Given the high quality of DNA prepared from sorted wheat chromosomes (Vrána et al. 2000), the technology is sufficiently mature to be applied to the physical mapping of DNA sequences, the targeted isolation of molecular markers and the construction of chromosome- and arm-specific DNA libraries. These approaches should facilitate the analysis of the complex genome of hexaploid wheat.

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