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Preparation of sub-genomic fractions enriched for particular chromosomes in polyploid wheat

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Abstract

Flow-sorted chromosomes have been used to simplify analyses of complex plant genomes. In bread wheat, majority of studies involve cultivar Chinese Spring, a genotype chosen for sequencing. Telosomic lines developed from this cultivar enable isolation by flow sorting chromosome arms, which represent less than 3.4 % of the genome. However, access to other wheat cultivars is needed to allow mapping and cloning useful genes. In these cultivars, cytogenetic stocks are not readily available and only one chromosome (3B) can be sorted. Remaining chromosomes form composite peaks on flow karyotypes and cannot be sorted. In order to overcome this difficulty, we tested a pragmatic approach in which composite chromosome peaks are dissected to smaller sections. The analysis of chromosome composition in sorted fractions confirmed feasibility of obtaining sub-genomic fractions comprising only a few chromosomes. Usually one of the chromosomes was more abundant and the frequencies of dominant chromosomes in sorted fractions ranged from 16 % (chromosome 7B) to 80 % (chromosome 2B). The enrichment factor, calculated as the relative proportion of chromosomal DNA in the wheat genome to the proportion of chromosomal DNA in a sorted fraction, ranged from 3.2-fold (7B) to 16.4-fold (5D). At least a 5-fold enrichment can be obtained for 17 out of 21 wheat chromosomes. Moreover, we show that 15 out of the 21 chromosomes can be sorted without being contaminated by their homoeologs. These observations provide opportunities for constructing sub-genomic large-insert DNA libraries, optical mapping, and targeted sequencing selected genome regions in various cultivars of wheat. The availability of fractions enriched for chromosomes of interest and free of contaminating homoeologs will increase the efficiency of research projects and reduce their costs as compared to whole genome approaches. The same methodology should be feasible in other plants where single chromosome types cannot be sorted.

Additional key words: chromosome sorting, complexity reduction, genome analysis.

Introduction

Evolution of higher plants and their nuclear genomes was accompanied by propagation of various classes of repetitive DNA, whole genome duplication (polyploidization), and interspecific hybridization, followed by polyploidization (allopolyploidization) (Bennetzen 2000, Van de Peer *et al*. 2009, Madlung 2013). As consequence, genomes of many species are large and characterized by sequence redundancy, hampering genome mapping, sequencing, and positional gene cloning.

A variety of approaches have been designed to overcome the difficulties. Although some of them (Paterson 2006, Ng *et al*. 2009) have not been used extensively, flow cytometric chromosome sorting enables dissection of nuclear genomes and has been used in a number of species comprising important legumes and cereals (Doležel *et al*. 2007, 2012). Applications include physical mapping and integration of genetic and physical maps (Kejnovský *et al*. 2001, Neumann *et al*. 2002, Vláčilová *et al*. 2002, Wenzl *et al*. 2010, Zatloukalová

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Abbreviations: DAPI - 4',6-diamidino-2-phenylindole; FISH - fluorescence *in situ* hybridization; FISHIS - FISH in suspension; PRINSES - primed *in situ* labelling *en* suspension; RIL - recombinant inbred line; STS - sequence-tagged site.

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et al. 2011), high resolution cytogenetic mapping (Valárik *et al*. 2004), targeted development of molecular markers from specific genome regions (Požárková *et al*. 2002), and construction of large-insert DNA libraries (Šafář *et al*. 2004, 2010, Paux *et al*. 2008). In wheat, the production of chromosome arm-specific BAC libraries has underpinned the international effort to sequence its huge genome (Feuillet and Eversole 2007, http://www.wheatgenome.org/). Shotgun sequencing chromosomal DNA by next generation methods is the latest addition to the portfolio of applications (Hernandez *et al*. 2012, Kantar *et al*. 2012, Martis *et al*. 2012, Shatalina *et al*. 2013, and the International Wheat Genome Sequencing Consortium, IWGSC, Science 345: 1251788, 2014).

The impact that flow-sorted chromosomes made in genomics of some species (chromosome genomics) generates interest to apply it more broadly. However, a bottleneck is the inability to discriminate particular chromosomes. The primary parameter according to which chromosomes are classified using flow cytometry is fluorescence of stained DNA. If the intensity of fluorescence (corresponding to a relative DNA content) of a chromosome is the same or very close to other chromosome(s) in the karyotype, the chromosome cannot be sorted without contamination by other chromosome(s). Unfortunately, it is common that plant species have two or more chromosomes similar in size. Hexaploid bread wheat (*Triticum aestivum* L., 2n=6x=42) may serve as an extreme example: out of 21 chromosomes, only chromosome 3B can be sorted from lines with a wild type karyotype (Vrána *et al*. 2000, Fig. 1).

Chromosomal composition of individual peaks of the standard wheat flow karyotype was first described by Vrána *et al*. (2000). In a larger-scale study, Kubaláková *et al*. (2002) flow karyotyped more than 50 different wheat cultivars. Most of the lines had similar fluorescence profiles to the standard, but some exceptions were found. These deviations were either due to chromosome polymorphism or structural changes, which resulted in the size change of particular chromosomes and made their discrimination and sorting possible. Nevertheless, only a few additional chromosomes could be separated from these lines, therefore a different approach to sort all wheat chromosomes must be contrived.

The first successful way in resolving more chromosome types in human and animal flow cytometric experiments was simultaneous use of two DNA dyes with opposite specificity for A/T and G/C base pairs (Langlois *et al*. 1982). Unfortunately, in plants this has very little or no effect on distinguishing more chromosomes (Lee *et al*. 1997, Lucretti and Doležel 1997). Attractive approaches to discriminate particular chromosomes are techniques involving labelling specific DNA sequences prior to flow cytometry: fluorescence *in situ* hybridization (FISH) and primed *in situ* labelling *en* suspension (PRINSES) (Macas *et al*. 1995, Pich *et al*. 1995, Ma *et al*. 2005). To our knowledge, these methods were not used following their publication, most probably because labelling was not quantitative and the distributions of chromosomes labelled specifically and non-specifically overlapped (unpublished observations).

Recently, Giorgi *et al*. (2013) reported on specific labelling some microsatellite sequences on plant

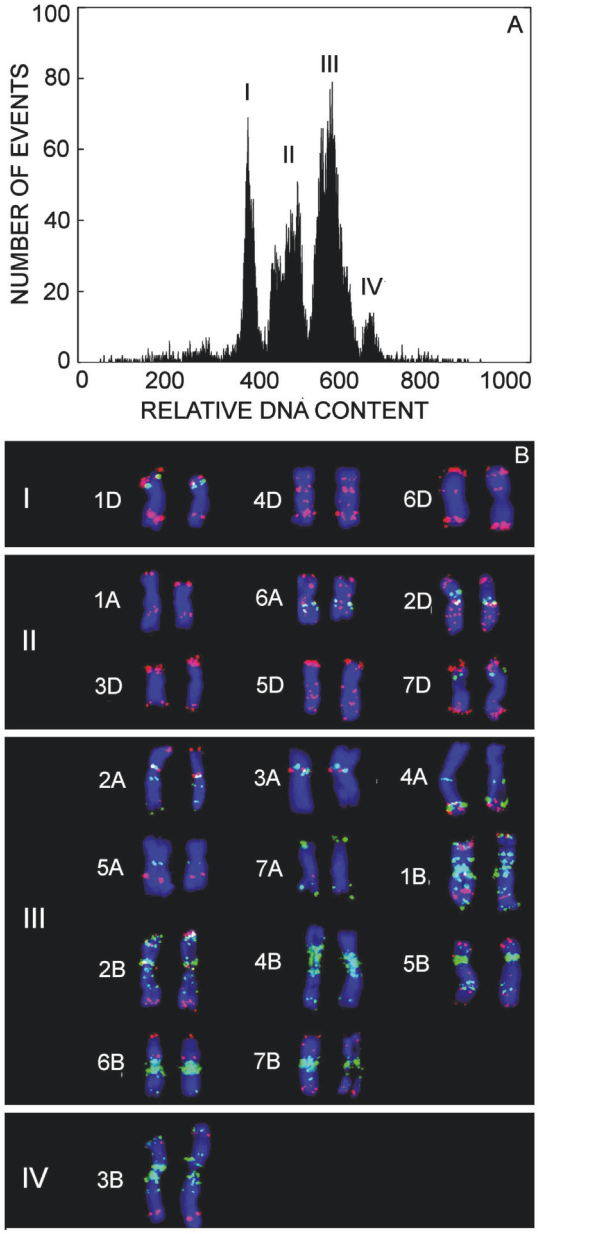

Fig. 1. Flow cytometric chromosome analysis and sorting in hexaploid bread wheat (2n=6x=42) cv. Chinese Spring. *A* - Distribution of relative DNA content (flow karyotype) obtained after the analysis of DAPI-stained mitotic chromosomes. The flow karyotype consisted of three composite peaks (I - III) representing groups of chromosomes, and a peak IV representing chromosome 3B. *B* - Chromosome content of each peak was determined using FISH on flow-sorted fractions with probes for GAA microsatellites (*yellow-green signals*) and *Afa* repeat (*red signals*). Chromosomes were counterstained by DAPI (*blue colour*). Two representative examples are shown for each chromosome.

chromosomes in suspension using a modified FISH protocol termed FISH in suspension (FISHIS) and showed its suitability to discriminate and flow-sort particular chromosomes in some species. The method will be useful in all cases where fluorescent labelling microsatellites can facilitate chromosome discrimination and where the FISHIS treatment is compatible with downstream applications of sorted chromosomes. At the moment, the biggest limitation of the method is that it works well with only a few types of repetitive sequences (unpublished results). Whole genome amplification of single chromosomes isolated using either microdissection or flow cytometric sorting (Ma *et al*. 2010, Yang *et al*. 2011) is another method which may overcome the difficulties due to inability to discriminate chromosomes of interest. Although the method has some limitations, for example, not the complete and uniform amplification of all sequences, pooling products of several independent amplification reactions of the same chromosome can increase the chromosome sequence coverage (Cápal *et al.* submitted).

Lack of suitable methods in plants led to the adoption of so far the most productive approach. It is based on exploiting cytogenetic stocks in which the size of some chromosomes has been changed so that they can be discriminated from other chromosomes and sorted. In some species, a wide range of cytogenetic stocks has been developed including chromosome translocations (Doležel and Lucretti 1995, Neumann *et al*. 1998, 2002, Kubaláková *et al*. 2002), deletions (Gill *et al*. 1999, Kubaláková *et al*. 2005), alien chromosome addition (Li *et al*. 2001, Kubaláková *et al*. 2003) and alien chromosome arms addition (Suchánková *et al*. 2006). A complete series of chromosome deletion lines in which chromosome arms are stably maintained as small telocentric chromosomes (telosomes) has been developed in bread wheat (Sears and Sears 1978). Similar stocks have been constructed also for barley and rye where individual chromosomes or arms are maintained in hexaploid wheat as stable alien addition lines. Flow cytometric sorting telosomes instead of whole chromosomes provides fractions of these huge genomes and enables localizing the position of centromere on genetic maps, which is otherwise difficult due to reduced recombination rate in pericentromeric regions (Mayer *et al*. 2011). However, there are two drawbacks of using cytogenetic stocks. First, they need to be developed and maintained, which is time consuming and laborious. Thus, a complete set of telosomic lines is available for only one cultivar of wheat – Chinese Spring. Second, the genetic background may limit the application of this approach when a chromosome DNA or library is needed from other genotypes than from which the stocks were developed.

In the light of above mentioned facts, we set out to explore a simple approach which is based on dissection of composite chromosome peaks to small regions. Although the present method does not have an ambition to provide pure chromosome fractions, it delivers fractions enriched for chromosomes of interest, which may be useful for various applications, and in particular for the construction of subgenomic large insert DNA libraries and optical mapping as well as for proteomics. In order to verify this approach, we chose hexaploid wheat where only one out of 21 chromosomes can be sorted from lines with the standard karyotype. We assessed the purity at which individual chromosomes can be sorted and discuss advantages and drawbacks of this method. We also give examples of the application of this simple approach using chromosome 4A as model.

Material and methods

Plants: Seeds of hexaploid bread wheat (*Triticum aestivum* L., 2n=6x=42) cvs. Chinese Spring and Renan with standard karyotypes were kindly provided by Dr. P. Sourdille (INRA, Clermont-Ferrand, France). Seeds of bread wheat cv. Tähti were kindly provided by Dr. K. Järve (the Tallinn University, Estonia). Seeds of double ditelosomic line 4A of cv. Chinese Spring (2n=40+2t4AS+2t4AL) were kindly provided by Prof. B.S. Gill (the Kansas State University, Manhattan, USA). Chinese Spring is a model wheat cultivar selected by the International Wheat Genome Sequencing Consortium (IWGSC) as genotype to be fully sequenced (Feuillet and Eversole 2007, http://www.wheatgenome.org/). Renan is a wheat cultivar selected by IWGSC together with Chinese Spring as parents for the development of a reference recombinant inbred line (RIL) genetic mapping population. The cv. Tähti was chosen because its chromosome 4A bears a *QPm-tut-4A* gene providing resistance to powdery mildew (Jakobson *et al*. 2012), and we have been involved in a project on cloning this gene. The ability to prepare chromosome 4A enriched fractions as described in the present work greatly aided the project. We have developed a set of gene-based molecular markers specific for 4A chromosomes of cvs. Tähti and Chinese Spring, and genomic locations of the markers were verified genetically and physically prior to this study.

Preparation of suspensions of intact mitotic chromosomes from synchronized root meristems was performed according to Vrána *et al*. (2000, 2012). Briefly, seeds were germinated in the dark at a temperature of 25 ± 0.5 °C on moistened filter paper in glass Petri dishes for 3 d to achieve an optimal root length (2 - 3 cm). Root tip cells were synchronized in the dark at 25 °C in a 2 mM hydroxyurea solution for 18 h, and 4.5 h after the release from the hydroxyurea block, dividing cells were accumulated at metaphase by a treatment with 2.5 mM amiprophosmethyl for 2 h, followed by an overnight treatment in ice water. Then the

roots were fixed in 2 % (v/v) formaldehyde in Tris buffer at 5 °C for 20 min. For 1 sample, 60 root tips were cut and homogenized using a *Polytron PT1300D* homogenizer (*Kinematica AG*, Littau, Switzerland) at 20 000 rpm for 13 s. The crude suspension was passed through a 20 μm pore size nylon mesh filter just before use to remove tissue debris and big clumps and stained with 4',6-diamidino-2-phenylindole (DAPI) to a final concentration of 2 μg cm^{-3}.

Flow cytometric analysis and sorting was carried out on a *FACSAria II SORP* high speed flow sorter (*BD Biosciences*, San Jose, USA) equipped with two lasers. A blue laser (488 nm, 100 mW) was used for analysis of light scatter (FSC, SSC), and an UV laser (355 nm, 100 mW) was used for detection of DAPI fluorescence through a 450/50 band pass filter. The alignment of the instrument was checked each day prior to sorting using *BD Cytometer Setup and Tracking Beads* (*BD Biosciences*). Coefficient of variation (CV) of bead signals in the DAPI detector ranged from 1.55 to 1.75 %. Dot plots of FSC/DAPI-A (forward scatter *vs*. DAPI fluorescence pulse area) and DAPI-W/DAPI-A (DAPI fluorescence pulse width *vs*. DAPI fluorescence pulse area) were used to discriminate single chromosomes from debris, clumps, chromatids, and chromosome doublets. In order to assure reproducibility of chromosome sort region positions, composite peak I of the bread wheat flow karyotype was used as marker and its mean was kept on the same channel throughout sorting by manual adjustment of the PMT voltage for the DAPI detector, as needed.

For sorting chromosomes from cv. Chinese Spring, sort windows were set on a dot plot of DAPI-W/DAPI-A. Identically spaced regions were used for sorting chromosomes from each of the three composite peaks I, II, and III. The number of sort regions in each composite peak corresponded to the number of chromosomes in the peak (*i.e.*, 3 regions in peak I, 6 regions in peak II, and 11 regions in peak III). This setting assumed an even distribution of chromosomes across the width of the composite peaks, which may not be the case. However, this was considered a good starting point, as a sort window width and position may be adjusted based on the analysis of sorted chromosome fractions by FISH. For FISH, 1 000 chromosomes were sorted from each partition onto a microscopic slide into 0.01 cm^3 drop of a PRINS buffer supplemented with 2.5 % (m/v) sucrose (Doležel *et al*. 1999). To assure reproducibility, the experiments were repeated on three different days and average frequencies of chromosomes were determined in the sorted fractions.

For sorting chromosome 4A from cvs. Tähti and Renan, composite peak III was divided into 11 equal sort regions and sorted onto slides as described above. For PCR, 500 chromosomes were sorted from each region into PCR tubes containing 0.01 cm^3 of deionized water. For each region, 10 PCR tubes were sorted in 3 independent experiments. For FISH, chromosomes from individual fractions of peak III were sorted in the same way as in the previous experiment.

The long arm of chromosome 4A (4AL) was isolated as telocentric chromosome from double ditelosomic line 4A of cv. Chinese Spring. For PCR, 1000 telosomes were sorted into PCR tubes containing 0.01 cm^3 of deionized water.

FISH: Chromosomes sorted from individual sort regions were identified using FISH according to Kubaláková *et al*. (2003) using probes for Sc119.2 and *Afa* repeats and GAA microsatellite. A digoxigenin-labelled probe for GAA microsatellites was prepared using PCR with $(GAA)_7$ and $(CCT)_7$ primers, and a 260-bp fragment of the *Afa* family repeat was prepared using PCR with primers AS-A and AS-B (50) and wheat (genomic) DNA as template. The Sc119.2 probe was prepared using PCR with primers M13 and DNA from plasmid pSc119.2 (Bedbrook *et al*. 1980) as template. The sites of digoxigenin-labelled probe for GAA microsatellites were detected using anti-digoxigenin-FITC raised in sheep (*Roche Molecular Biochemicals*, Mannheim, Germany). A biotin-labelled probe for the *Afa* family repeat was detected using Cy3-labelled streptavidin. Chromosome preparations were counterstained with 0.2 μg cm^{-3} DAPI and mounted in a *Vectashield* antifade solution (*Vector Laboratories*, Burlingame, USA) and the preparations were evaluated using an *Olympus BX60* (Tokyo, Japan) microscope. The images of DAPI, fluorescein, and Cy3 fluorescence were acquired separately with a *b/w CCD* camera, which was interfaced to a PC running the *ISIS* software (*Metasystems*, Altlussheim, Germany).

PCR with chromosome-specific markers: We used two sequence-tagged site (STS) markers developed in our laboratory (*owm*, the Olomouc wheat marker) to identify sort regions containing chromosome 4A and evaluate feasibility of PCR on the sorting regions. The markers were derived from Chinese Spring genomic DNA during an ongoing positional cloning project of a powdery mildew resistance gene introgressed to cv. Tähti from *T. militinae* (Jacobson *et al*. 2012). Gene-based STS marker *owm*31 (forward primer: CGAGCTCGACAT GAACCTG, reverse primer: AGCAACCCATCCAACC ATT) amplifies two bands with genomic DNA: a 205 bp band specific to chromosomes 7B and 7D, and a 220 bp fragment specific to wheat chromosomes 4A and 4B (unpublished). The second marker *owm*39 (F: TGG CCCTATCCAAGGTTCTA, R: TGGCCCTATCCA AGGTTCTA) produces a 400 bp band exclusively specific for chromosome 4A (unpublished).

PCR was carried out on 500 chromosomes sorted from each of the 11 regions of composite peak III. Wheat genomic DNA (5 ng) and MDA-amplified DNA of chromosome 4A (Šimková *et al*. 2008) were used as controls. A PCR reaction mix (2×10^{-2} cm^3) consisted of a DNA template, 200 μM dNTPs, 1 μM each primer, 1× PCR buffer P-511 (*Fermentas*, Vilnius, Lithuania), 0.01 % (m/v) Cresol Red, 1.5 % (m/v) sucrose, and 0.8 U

of *Taq* polymerase (*Fermentas*). The reaction was carried out on a *PTC-200* thermal cycler (*Bio-Rad*, Hercules, USA) as follows: 95 °C for 5 min, 40 × [95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s], and a final extension at 72 °C for 10 min. PCR products were separated on a 3.5 % (m/v) native polyacrylamide gel at 350 V for 60 min on a *MegaGel* system (*S.B.S. Scientific*, San Diego, USA). The gel was soaked with ethidium bromide (0.0002 %, m/v) during gel pre-run (at 300 V for 60 min), and DNA fragments were visualized using a gel documentation system *Chemigenius* (*Syngene*, Frederick, USA).

Results

The karyotype of bread wheat (*Triticum aestivum*; 2n=6x=42) consists of three homoeologous sets of chromosomes (A, B, and D), each with seven chromosomes. The flow cytometric analysis of chromosomes isolated from cv. Chinese Spring with the standard karyotype resulted in a flow karyotype comprising four peaks (Fig. 1). After sorting particles from the four peaks onto microscopic slides and subsequent analysis using FISH with probes which give diagnostic fluorescent labelling pattern, the chromosomal composition of individual peaks was established (Fig. 1). There were 3 chromosomes in composite peak I (1D, 4D, 6D), 6 chromosomes were assigned to peak II (1A, 6A, 2D, 3D, 5D, 7D) and 11 chromosomes were sorted from peak III (2A, 3A, 4A, 5A, 7A, 1B, 2B, 4B, 5B, 6B, 7B). The largest wheat chromosome 3B formed a single peak to the right of composite peak III.

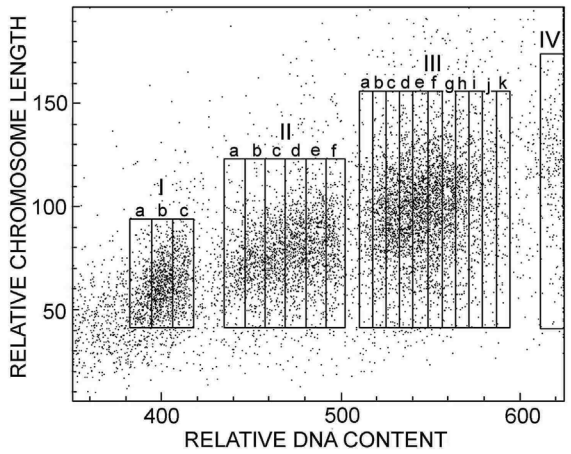

Fig. 2. A detail of a dot-plot obtained after the analysis of chromosomes of cv. Chinese Spring showing positions of sort regions. The number and hence width of sort windows in composite peaks was determined by the number of chromosomes represented by peaks. Each sort window is indicated by a combination of *Greek numerals* (the number of the peak) and *small case letters* (the position in the peak). The position of the first peak (I) was used as "anchor" in consecutive sorting experiments.

After the initial analysis, which confirmed our previous observations (Vrána *et al*. 2000, Kubaláková *et al*. 2002), we divided the three composite peaks into equally sized partitions (Fig. 2). Equal spacing and sizes of the sections were chosen to obtain initial information about approximate positions of individual chromosomes. The rationale was that once the location of a chromosome is determined, the position of a sort region can be optimized individually. In these experiments, composite peak I was used as a marker and its mean was continually kept on the same channel.

As expected, each of the 21 wheat chromosomes, except chromosome 3B, which forms its own peak was present in several fractions sorted from a group of neighbouring sort regions (Table 1 Suppl.), reflecting the width of its frequency distribution. The frequencies of the most abundant chromosomes in sort regions ranged from 16 % (chromosome 7B) to 80 % (chromosome 2B). Generally, the best purities were achieved for chromosomes on the periphery of the composite peaks. For example, chromosome 4D could be sorted from peak I with a purity of 55 %, chromosome 5D could be sorted from peak II with a 72 % purity, and chromosome 2B was sorted from peak III with a purity of 80 %. The lowest purities were observed for chromosomes in the middle of peak III (21 % for 3A and 16 % for 7B). This is a logical consequence of multiple overlapping chromosome peaks. Chromosome 3B was represented by a separate peak and could be sorted with purity higher than 94 %.

The chromosome content of individual sort regions and average purities determined in chromosome fractions sorted in three independent experiments are summarized in Table 1 Suppl. There were small differences in chromosome frequencies between the replicate experiments (data not shown). However, the differences did not result in the change of sort windows with the highest frequency of respective chromosomes.

In order to quantify enrichment in terms of the quantity of DNA from a particular chromosome, we determined an enrichment factor by comparing the relative proportion of a chromosome in the whole wheat genome to the proportion of chromosome DNA in a sorted fraction. Molecular chromosome sizes were taken from Šafář *et al*. (2010). The enrichment factor determined this way ranged from 3.2-fold (7B) to 16.4-fold (5D) (Fig. 3). At least a 5-fold enrichment was obtained for 17 out of the 21 chromosomes. If the number of chromosomes was considered in each fraction in comparison with the complete genome (21 chromosomes), a decrease in sample complexity ranged from 2.1-fold for region IIIe with 10 chromosomes to 10.5-fold for region IV with only 2 chromosomes.

Our analysis of sorted chromosome fractions by FISH reveals that 14 wheat chromosomes could be sorted

without being contaminated by their homoeologous counterparts. Moreover, all three homoeologous chromosomes could be completely separated in chromosome groups one, three, four, and six (Fig. 4). This is a consequence of differences in a relative DAPI fluorescence between the homoeologs and hence non-overlapping frequency distributions (Fig. 4). For three chromosomes (7A, 7B, and 7D), the contamination by homoeologs fell below 5 %, and only for one chromosome (2B), the contamination was above 10 %. The analysis of frequency distributions of chromosomes contaminated by homoeologs indicates that for some of the chromosomes (*e.g.*, 2B), it will not be possible to obtain highly enriched fractions without contamination by homoeologs (Fig. 5).

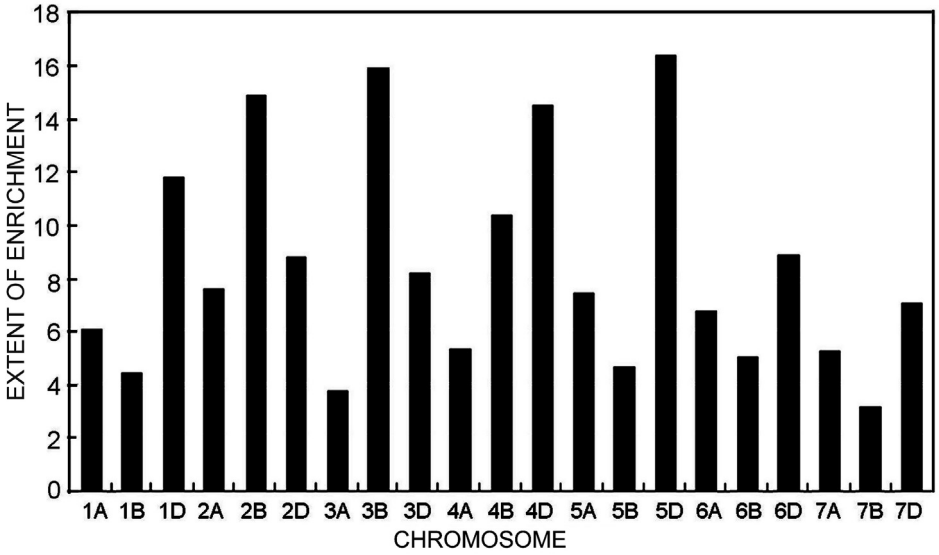

Fig. 3. The extent of enrichment obtained for 21 chromosomes of bread wheat cv. Chinese Spring after sorting from regions as shown in Fig. 2. The enrichment was estimated after comparing relative proportion of a chromosome in the whole wheat genome to proportion of chromosome DNA in flow-sorted fraction.

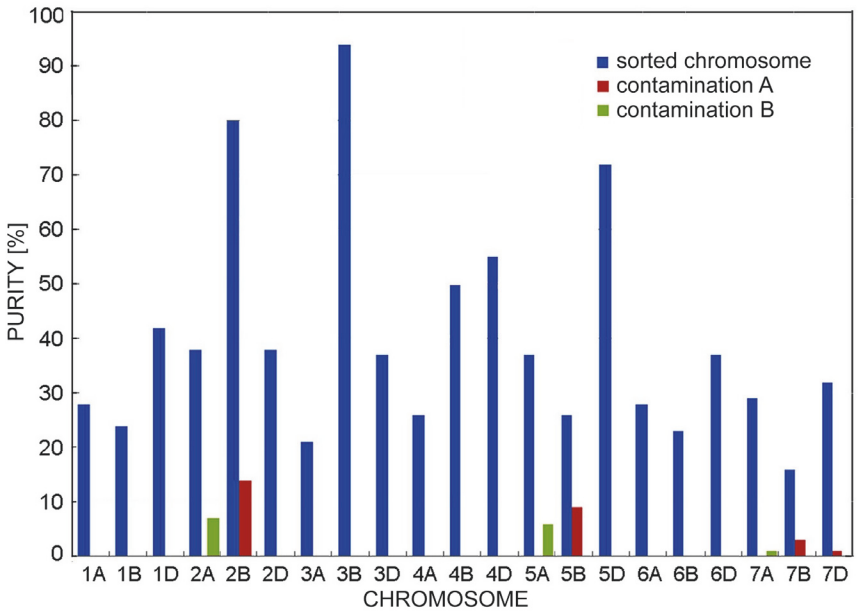

Fig. 4. Contamination [%] of sorted chromosome fractions by homoeologs in cv. Chinese Spring. Purities in flow-sorted chromosome fractions are marked in *blue*, whereas the frequency of contaminating chromosomes from A genome in *orange* and B genome in *green*, respectively. Chromosomes from D genome did not contaminate their homoeologous counterparts.

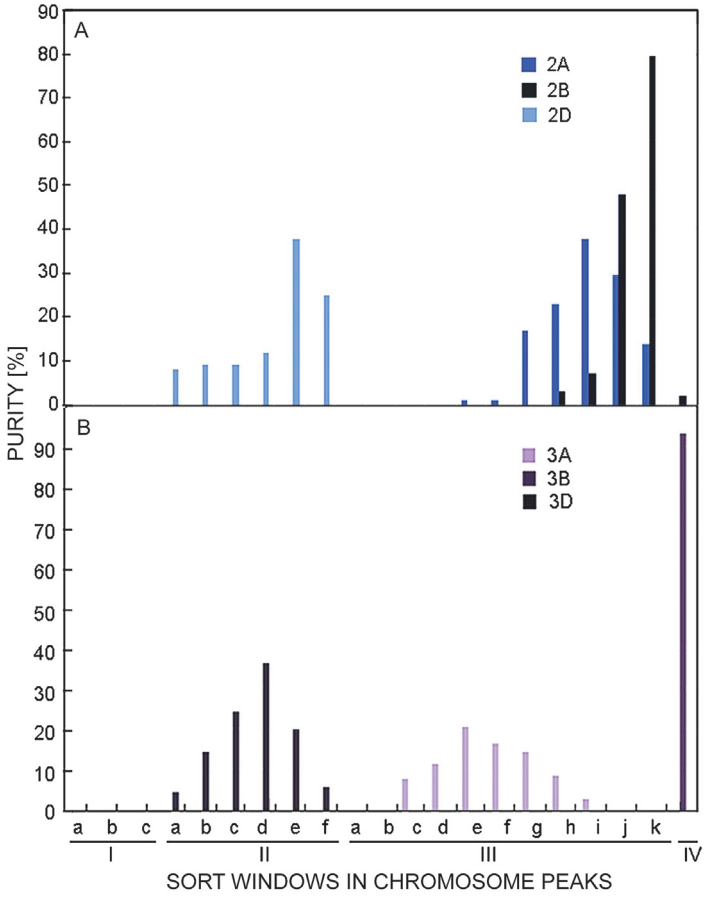

Fig. 5. Two extreme examples of distribution of relative chromosome DNA content and of contamination of sorted chromosomes by homoeologs in selected groups as observed in cv. Chinese Spring. *A* - Homoeologous group 2: distributions of chromosomes 2A and 2B overlap and their sort fractions are cross-contaminated. Only chromosome 2D could be sorted without being contaminated by homoeologs. *B* - Homoeologous group 3: chromosome distributions do not overlap, and all three chromosomes could be sorted without contamination by homoeologs.

With some margin error, the analysis of fractions sorted from peak III of cv. Renan confirmed the expected location of chromosome 4A in sort region "h", which was identical to the location of chromosome 4A from cv. Chinese Spring. A different position than expected was observed for chromosome 4A in cv. Tähti, as the fraction with the highest abundance (29 %) could be obtained from sort region "d" of composite peak III (Table 2 Suppl.). The reason for the difference could be a smaller chromosome size and hence a smaller DNA content. This hypothesis was confirmed by FISH with a probe for pSc119.2, which revealed the absence of the sequence cluster at the terminal part of the long arm of 4A (Fig. 6).

In addition to FISH, the presence of chromosome 4A in the sorted fractions was assessed using two gene-based PCR markers *owm*31 and *owm*39. The physical location of *owm31* and *owm*39 loci from which bands were produced was verified using a panel of MDA-amplified chromosomal arms of all wheat ones (unpublished). The genetic location of the markers was verified using two recombinant mapping populations from the crosses of introgression line 8.1 with cv. Tähti and cv. Chinese Spring (Jakobson *et al*. 2012). The *owm*31 marker amplified a 220 bp fragment from chromosomes 4A and 4B and also a 200 bp fragment from chromosomes 7D and 7B. These four chromosomes were present in composite peak III (Fig. 1). As the 4B and 4A-specific 220 bp band was identified in sort regions a, b, and d – k, respectively (Fig. 7*A*), the PCR results confirmed the location of chromosomes 4A and 4B as determined by FISH (Table 1 Suppl.). PCR products specific for chromosomes 7B and 7D (200 bp) were identified in sort regions a, b, c and h, i, j – again confirming the FISH analyses. In order to verify the identity of chromosome 4A, the *owm*39 marker specific to the Chinese Spring 4AL chromosome arm was used. The marker amplified a 400 bp band (Fig. 7*B*) in sort region h, which confirmed both results.

Discussion

We present a pragmatic approach for preparation of subgenomic fractions enriched for particular chromosomes in polyploid wheat. This method is attractive for all species where chromosomes cannot be discriminated from each other as their distributions of relative DNA content overlap and form composite peaks on flow karyotypes. This method may be of help either when cytogenetic stocks (Doležel *et al*. 2012) and fluorescent labelling DNA microsatellites using FISHIS (Giorgi *et al*. 2013) would not be applicable, or when low purity sorting is allowed by experimental purposes.

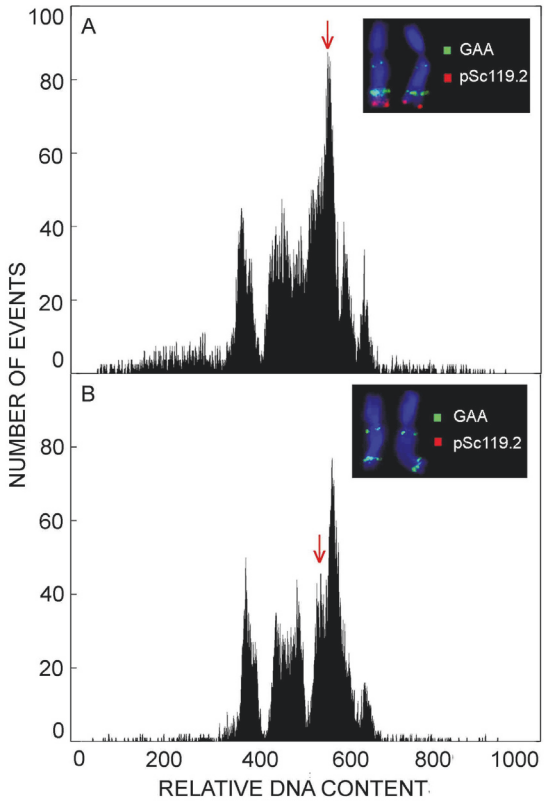

Fig. 6. Flow karyotypes of bread wheat cvs. Renan (*A*) and Tähti (*B*). Positions of chromosome 4A-richest sort region in composite peaks III in both cultivars are indicated by *arrows*. *Insets*: chromosome 4A was identified in sorted fractions after FISH with probes for GAA microsatellites (*yellow-green signals*) and pSc119.2 repeat (*red signals*). Chromosomes were counterstained with DAPI (*blue colour*). Note the absence of pSc119.2 sequence cluster (*red*) at terminal part of long arm of 4A in cv. Tähti.

In fact, the present approach and the other two methods are complementary. The use of cytogenetic stocks, such as telosomic lines, enables the preparation of pure fractions, and an advantage is that single arms are sorted (Doležel *et al*. 2007, 2012). This results in a larger reduction of sample complexity as compared to sorting intact chromosomes and enables mapping centromere position (Mayer *et al*. 2011). However, the telosomic lines are available in a limited number of plant species and their development is laborious and time consuming. Sorting chromosomes after FISHIS (Giorgi *et al*. 2013) allows sorting intact chromosomes. However, the latter approach is not suitable for downstream proteomic analyses as it includes a denaturation step and becomes laborious if many millions of chromosomes have to be sorted, *e.g.*, for the construction of large insert DNA libraries (Šafář *et al*. 2010) and optical mapping (Zhou *et al*. 2007). In contrast, the present approach is simple, generally applicable, and does not require special cytogenetic stocks and/or a special treatment of chromosomes prior to sorting, which could compromise their suitability for downstream applications. As the sort window needs to be rather narrow in order to reduce sorting other chromosomes with a similar DAPI fluorescence, the present approach results in lower sort rates as compared to a situation where the chromosome of interest is clearly discriminated from other chromosomes. This is because some of target chromosomes are not sorted as their fluorescence exceeds sort window limits. The sorted fractions are also less pure as compared to the other two methods. On the other hand, the sorted chromosomes are suitable for several applications in genomics (Wenzl *et al*. 2010, Mayer *et al*. 2011, Vrána *et al*. 2012) and proteomics (Petrovská *et al*. 2014) not requiring high purity sorting.

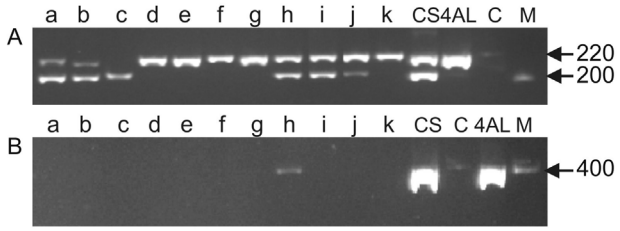

Fig. 7. Amplification patterns obtained using PCR with markers *owm*31 and *owm*39 on chromosome fractions flow-sorted from different regions of composite peak III of cv. Chinese Spring. *Lane labels* correspond to the partitions of peak III as marked in Table 1 Suppl. and Fig. 2. *A* - Marker *owm*31 identifies position of four chromosomes (4A, 4B, 7B, and 7D). Chromosomes 4A and 4B are represented by a 220 bp band in *lanes* d - k and a - b, respectively. Chromosomes 7B and 7D are represented by a 205 bp band in *lanes* h - j and a - c, respectively. *B* - Marker *owm*39 is specific for chromosome 4A. Note that the product is observed only in the fraction most enriched for 4A (*lane* h) as the marker requires a higher amount of template. Additional *lanes* are marked as CS - genomic DNA of cv. Chinese Spring; 4AL - long arm of chromosome 4A of cv. Chinese Spring (flow-sorted as telosome from double ditelosomic line 4A); C - negative control; M - size marker.

We choose hexaploid bread wheat as model for this project as only one chromosome (3B) can be discriminated in genotypes with the standard karyotype, whereas other chromosomes are represented by three composite peaks. Our results show that it was possible to

sort chromosomes of interest from composite chromosome peaks to obtain defined sub-genomic fractions. As compared to a whole genome analysis, the fractions were enriched 3.2- to 16.4-fold for chromosomes of interest and the frequency of the most abundant chromosomes in sorted fractions ranged from 16 to 80 %. Thus, our approach facilitates preparation of samples with reduced complexity to simplify molecular genomic analyses of complex and polyploid plant genomes. It should be noted that the data on chromosome purities and the degrees of enrichment as obtained in the present work are specific for hexaploid wheat and its cv. Chinese Spring. These parameters are species- and genotype-specific as we show here in wheat cv. Tähti and will have to be experimentally verified. The optimization of sort window position and width is rather straightforward and involves determination of chromosome content in fractions sorted using different sort windows settings. Evaluation is done microscopically after FISH with probes which give chromosome-specific labelling patterns. Typically, a few sort runs followed by the evaluation of the sorted fractions is sufficient to define an optimal sort window position.

Apart from the reduction of sample complexity, an attractive feature of chromosome sorting in allopolyploid species is the potential of obtaining fractions of chromosomes free of contaminating homoeologous chromosomes which have a similar molecular composition. However, as the homoeologs originate from different species, they may differ in relative fluorescence as observed in bread wheat (Vrána *et al*. 2000). We show that in wheat with seven groups of homoeologous chromosomes, 15 chromosomes could be sorted without being contaminated by their homoeologs. For chromosome group 7, the contamination by homoeologs was less than 5 %, and only for chromosome 2B, the contamination exceeded 10 %. The ability to prepare chromosome fractions free of homoeologs will greatly facilitate development of genome- and chromosome-specific molecular markers and provide an elegant approach to map DNA sequences to individual chromosomes of particular (sub)genomes. This work could be facilitated by the availability of chromosome shotgun sequences, obtained in cv. Chinese Spring (www.wheatgenome. org/), which could be used as reference for other genotypes.

Following the evaluation of a potential of flow sorting to prepare enriched chromosome fractions in a model genotype of bread wheat, we explored possibilities for sorting chromosome 4A from two other cultivars of wheat. Since our laboratory coordinates construction of ready-to-sequence physical map of chromosome 4A, and we have two positional cloning projects on chromosomal arm 4AL, we developed a panel of 4A-specific markers for which genetic and physical locations were verified (unpublished). Taking advantage of the availability of 4A-specific markers, and the fact that the chromosome is found in the middle of the most complex composite peak III of the wheat flow karyotype, we chose this chromosome as model to verify the approach proposed in this work using wheat cvs. Renan and Tähti. The cultivar Renan has been used as the second parent to produce a large F8 mapping population after crossing with cv. Chinese Spring (Feuillet, pers. comm.). The ability to purify chromosomes from cv. Chinese Spring and cv. Renan would enable a high-throughput development of polymorphic DNA markers specific for most of the chromosomes. The position of chromosome 4A in the composite peak III of cv. Renan was similar to that in cv. Chinese Spring. However, its position was different in cv. Tähti as verified by PCR and FISH. This observation confirms and expands the results of our earlier study in which we observed chromosome polymorphism and variability of flow karyotypes after analyzing a large set of wheat cultivars (Kubaláková *et al*. 2002). The variation was attributed to polymorphism in heterochromatin bands and chromosome size.

In summary, the results presented widen the use of chromosome flow sorting in polyploid wheat and in wheat cultivars other than the model cultivar Chinese Spring. This is important as cv. Chinese Spring is known not to carry some genes of interest, which can only be mapped and cloned from other cultivars (Stein *et al*. 2000, Feuillet *et al*. 2003, Huang *et al*. 2003, Uauy *et al*. 2006). The availability of fractions enriched for chromosomes of interest and possibly free of contaminating homoeologous chromosomes will increase the efficiency of research projects and reduce their cost as compared to the whole genome approach. The applications may involve construction of large-insert DNA libraries enriched for a chromosome of interest (*e.g*., custom BAC libraries), optical mapping, proteomic analyses, and production of amplified DNA enriched for a chromosome of interest for next-generation sequencing, developing DNA markers, and mapping. Moreover, the present results indicate that it should be possible to use the same approach to facilitate chromosome genomics in other species where two or more chromosomes do not differ significantly in size and cannot be discriminated using other parameters, such as light scatter and after fluorescent labelling microsatellite DNA.

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