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Generating resources for genomics of wheat homoeologous chromosome group 3: 3AS- and 3DS-specific BAC libraries

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ABSTRACT

In bread wheat comprehensive genome analysis is complicated by several factors such as a very large genome (~17 Gbp), its polyploid nature and a high repetitive sequence content. Using flow cytometry allows to dissect the wheat giant genome to defined parts mainly its chromosomes and chromosome-arms and can help to solve this challenge by enabling the chromosome-based strategy. Flow-sorted chromosomes were used already to construct several grain BAC libraries including libraries from chromosome 3B and chromosome arms 1BS and 1RS. They became valuable resources for wheat physical mapping and positional cloning, and they had an insert size of 75-85 kb, as average. This insert size was a consequence of a limited amount of DNA obtained after a time-consuming sorting which enabled only one size-selection step. In the present work we compare the construction of the 3AS BAC library in respect to our new improved protocol which made the second size-selection step feasible and which was applied for the construction of a BAC library specific for chromosome arm 3DS, which showed an average insert size reaching 110 kb. A minimal tiling path of chromosome 3B has already been established. Construction of the 3AS- and 3DS-specific BAC libraries represent significant step toward completing BAC resources for the homoeologues chromosome group 3 of wheat and accelerate the development of sequence-ready physical contig maps and gene cloning, as well as comparative analyses aiming at revealing the genome changes accompanying the evolution of homoeologous chromosome group 3.

Key words: BAC library, Flow sorting, Homoeologous chromosomes, Physical map, Polyploidy

INTRODUCTION

Bread wheat (*Triticum aestivum* L., 2n = 6x = 42) is one of the most important crops providing staple food for 35% of the population and 20% of the calories consumed (http://www.CIMMYT.org/). Bread wheat is grown in all areas of temperate zones and its global significance can be compared only with rice. Maintaining stable yield of wheat is annually challenged by attacks of a large variety of diseases and pests and changes in climate. Wheat production in sufficient amount and quality become the challenge of the 21st century for scientist, breeders and farmers.

Being an allohexaploid species, *T. aestivum* posses a genome comprising three closely related sub-genomes A, B and D. This results in large genome size and great genetic redundancy. One copy of the wheat genome (1C) contains about 17 billion base pairs (BENNETT and SMITH, 1991). In fact, each of the wheat chromosomes is larger than the entire rice genome (*Oryza sativa* L., 489 Mbp/1C; BENNETT and SMITH, 1991) and the whole genome is more than hundred-fold that of Arabidopsis (*A. thaliana*, 157 Mbp/1C; BEN-

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NETT *et al.*, 2003). These unique features pose great challenges for gene discovery, genome sequencing and wheat improvement in general.

The development of appropriate strategies and technologies to decipher the wheat genome was going on during last decade. Among other, selective sequencing of random Bacterial Artificial Chromosome (BAC) clones from gene-rich regions, shot-gun sequencing of "gene-enriched" DNA obtained after genome filtration was suggested (GILL *et al.*, 2004). Powerful shot-gun sequencing technologies such as 454, and/or Solexa and solid systems which became available recently reduce sequencing costs and permit whole genome sequencing. However, regardless of which strategy is chosen, a robust physical map will be required to master the assembling of the whole wheat genome sequence. Physical maps consisting of overlapping large-insert DNA clones such as BACs are necessary not only for the sequencing approaches but to greatly accelerate positional gene cloning and crop improvement.

Several genomic BAC libraries have been constructed for bread wheat (LIU *et al.*, 2000, MA *et al.*, 2000, ALLOUIS *et al.*, 2003, NILMALGODA *et al.*, 2003, SHEN *et al.*, 2005). To construct the physical map of the whole genome, 1 to 2 million BAC clones will have to be fingerprinted. Although it is possible to fingerprint such large numbers of clones with the automated fingerprinting technique (LUO *et al.*, 2003), their specific assembly into contigs, and anchoring to genetic maps represent a daunting task, especially in the presence of three homeologous genomes.

This limitation could be overcome by the "chromosome-based strategy" (DOLEZEL *et al.*, 2007) which takes a vantage of the possibility to dissect wheat genome to defined parts (chromosomes, chromosome-arms) using the available wheat aneuploid mutant collection (http://www.K-state.edu/wgrc/germplasm/stocks/stocks.html) and flow sorting. VRÁNA *et al.*, (2000) and KUBALÁKOVÁ *et al.*, (2002, 2005) developed procedures for purification of individual chromosomes and chromosome arms of wheat by flow cytometry. As the relative size of wheat chromosomes and their arms range from 3.6 to 5.8% and from 1.3 to 3.4% of the genome, respectively (GILL *et al.*, 1991), this approach offers a significant reduction in complexity (with the largest wheat chromosome 3B representing 1Gb) thereby facilitating the development of physical maps in a step-wise manner, isolating one chromosome (arm) at a time, and providing the opportunity to divide the task between laboratories in an internationally coordinated effort.

The use of chromosome BAC libraries can minimize also problems related to the mirroring of isolated gene regions in paralogous loci caused by large-scale genome duplications detected in crop genomes (GUYOT and KELLER 2004, VALÁRIK *et al.*, 2006).

ŠAFÁR *et al.*, (2004) were the first to demonstrate that DNA of flow-sorted wheat chromosomes is suitable for construction of BAC libraries. Subsequently, a composite library specific for chromosomes 1D, 4D and 6D (JANDA *et al.*, 2004), and a library specific for the short arm of wheat chromosome 1B (1BS) was constructed (JANDA *et al.*, 2006). The same approach has been used to construct a BAC library from the short arm of rye chromosome 1R (ŠIMKOVÁ *et al.*, 2008).

Chromosome BAC libraries consist of 10^4 – 10^5 BAC clones only, which greatly facilitates their management and construction of physical maps and contig assembly. Recently, results obtained with the development of a physical contig map of wheat chromosome 3B confirm the advantages of chromosome-based strategy (PAUX *et al.*, 2008).

In this paper we describe the construction and characterization of two BAC libraries specific for the short arm of chromosome 3A (3AS) and 3D (3DS) of wheat, which account for 2.1 and 1.9 per cent of the hexaploid wheat genome, respectively. These two new BAC libraries represent a significant step toward completing BAC resources for the homoeologues chromosome group 3 of wheat. In addition to facilitating positional gene cloning, these resources open avenues for comparative analysis of homoeologous wheat genomes. An improved procedure for the production of larger sized BAC inserts is also described.

MATERIALS AND METHODS

Plant material. Two double ditelosomic lines (Ddt) of hexaploid wheat *Triticum aestivum* L. cv. 'Chinese spring' (2n=40+2t3AS+2t3AL; 2n=40+2t3DS+2t3DL) carrying the short arms of chromosomes 3A (3AS)

and 3D (3DS) as telosomes were used. Seeds were germinated in the dark at 25±0.5 °C on moistened filter paper for 3 days to obtain root length around 2-3 cm. Approximately 7,000 seeds were germinated in batches of 30-35 for the preparation of 204 samples of chromosome suspensions which were further used to sort 3AS; 5,000 seeds were used for preparation of 144 samples of chromosome suspensions to sort 3DS.

Preparation of chromosome suspensions and flow sorting. Cell cycle synchronization and preparation of suspensions of intact chromosomes were performed according to VRÁNA *et al.* (2000). Briefly, each of the samples of chromosome suspension was prepared by mechanical homogenization of 25 formaldehyde-fixed meristem root tips in 1 mL ice-cold isolation buffer (IB) of ŠIMKOVÁ *et al.* (2003). Chromosomes in suspension were stained by 2 µg/mL DAPI (4',6-diamidino-2-phenylindole) and analyzed using a FACSVantage flow cytometer (Becton Dickinson, San José, USA) equipped with an argon UV laser set to multiline UV and running at 300mW output power. The chromosome arms were sorted in aliquots of 2×10^5 into 320 µL of 1.5×IB. The purity in sorted fractions was checked regularly by sorting 2000 particles into 15µL drop of PRINS buffer supplemented with 5% sucrose on a microscope slide. After air-drying, sorted chromosomes were identified using fluorescence *in situ* hybridization (FISH) with probes for the GAA microsatellite and *Afa* repeats following the procedure already described in KUBALÁKOVÁ *et al.* (2002).

Construction of the 3AS-specific BAC library. Preparation of high molecular weight DNA and BAC library construction were performed as described previously (ŠAFÁR *et al.*, 2004). Briefly, each batch of 2×10^5 flow-sorted 3AS chromosomes was pelleted and the chromosomes were embedded in 15 µL of low melting point agarose (0.8%, w/v). In total, 25 agarose miniplugs representing 4.9×10^6 sorted chromosomes were prepared and used for DNA cloning. Chromosomal DNA was partially digested with *Hind*III and size-selected by pulsed field gel electrophoresis (PFGE) in 0.25×TBE buffer at 6V/cm, with a 1 – 40 s switch time ramp, angle 120°, for 12 hours and with a 2.5 – 5.5 s switch time ramp, angle 120°, for 6 hours at 14 °C. Three regions of the gel corresponding to approximately 75-100 kb ('C2 fraction'), 100-150 kb ('C3 fraction'), and 150-200 kb ('C4 fraction'), were excised, the HMW DNA was electroeluted and ligated into a dephosphorylated vector pIndigoBAC5 (Epicentre, Madison, USA) in molar ratio 1 to 4-6 (in BAC vector excess). Three ligations corresponding to fragment size were performed and incubated at 16 °C and after de-salting transformed into *Escherichia coli* ElectroMAX DH10B competent cells (Gibco BRL, Gaithersburg, USA) by electroporation. The library was ordered in 384-well microtitre plates filled with freezing medium (WOO *et al.*, 1994). The plates were incubated at 37 °C overnight and after their replication stored at –80 °C.

Construction of the 3DS-specific BAC library. The library was constructed according to the same protocol mentioned above. Two size selections by pulsed field gel electrophoresis (PFGE) were performed as a only one difference. Briefly, partially digested DNA was size-selected by PFGE in 1% *w/v* Gold SeaKem agarose (BMA) gel at 6 V/cm, 12.5 °C, in 0.25 × TBE for 17 h, with a 1.0–50 s switch time ramp and an angle of 120°. After electrophoresis, the region containing non-stained HMW DNA of approximately 100–300 kb (B=bottom 100-150kb, M=middle 150-200kb, T= top 200-300kb) was excised from the gel and subjected to a second round of size selection by PFGE in 0.9% *w/v* Gold SeaKem agarose gel at 6 V/cm, with 3 s of switch time and a 120∞ angle for 17 h. Subsequently, the procedure followed previously mentioned protocol.

Estimation of insert size of BAC clones. Two hundred BAC clones were randomly selected from all ligations and incubated overnight at 37 °C in 1.5 mL of 2YT medium (SAMBROOK *et al.*, 2001) containing 12.5 µg/mL chloramphenicol. BAC DNA was extracted by standard alkaline lysis method and digested with *Not*I restriction endonuclease (0.25 U/20 µL). PFGE was used to separate DNA fragments in 1% agarose gel in 0.5 × TBE buffer at 6V/cm, with a 5 – 15 s switch time ramp, angle 120°, for 15 hours at 14 °C. The insert sizes were estimated after comparison with a lambda size standard run in the same gel.

RESULTS AND DISCUSSION

The short arms of chromosomes 3A (3AS) and 3D (3DS) were purified from Ddt lines of hexaploid wheat *Triticum aestivum* L. cv. 'Chinese spring'. The work involved preparation of liquid suspensions of intact chromosomes from synchronized root tip meristems, staining with a DNA-specific fluorescent dye DAPI, and analysis of fluorescence intensity of stained chromosomes using flow cytometry. We obtained reproducible distributions of relative fluorescence intensity ('flow karyotypes'), which were characterized by six peaks (Figure 1). We confirmed chromosome content of each peak by sorting particles from each peak onto microscope slides and performing fluorescence *in situ* hybridization (FISH) with probes for GAA microsatellite and the *Afa* repeat. The two probes facilitated identification of any chromosome arm in hexaploid wheat (PEDERSEN and LANGRIDGE, 1997). We established that peaks I – III represented various chromosomes and that the small peak to the right of peak III represented chromosome 3B (VRÁNA *et al.*, 2000, KUBALÁKOVÁ *et al.*, 2002). The leftmost peak was found to represent the short arm of chromosome 3AS (Figure 1), and the short arm of chromosome 3DS (Figure 2).

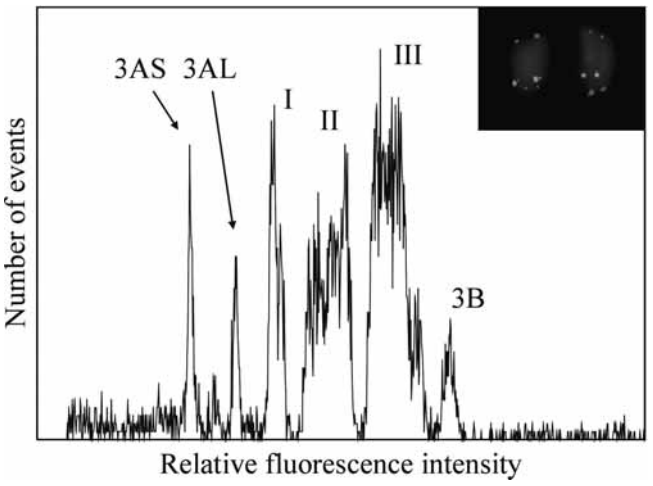

FIGURE 1 - Histogram of relative fluorescence intensity ("flow karyotype") obtained after flow cytometry analysis of DAPI-stained suspension of chromosomes prepared from double ditelosomic line 3A of wheat cultivar Chinese Spring. From the left to the right side, the flow karyotype consists of two peaks representing short (3AS) and large (3AL) chromosome arms of 3A, three composite peaks (I, II, and III) representing specific groups of chromosomes and a peak representing chromosome 3B. Examples of flow-sorted chromosome 3AS are shown in the insert after FISH with a probe for telomere repeats (red signals) and GAA microsatellite (green signals). The chromosomes were counterstained with DAPI (blue color).

Subsequently, we sorted 4.9 million copies of chromosome arm 3AS (Figure 1) representing approximately 3.6 µg DNA in batches of 2×10^5 and embedded them in 25 agarose plugs. The sorting exercise took 25 working days and approximately 7000 seeds were needed to prepare 204 samples of chromosome suspensions. We sorted 5.3 million copies of chromosome arms 3DS representing approximately 3.4 µg DNA in batches of 2×10^5 and embedded them in 26 agarose plugs. The sorting exercise took 18 working days and approximately 5,000 seeds were needed to prepare 144 samples of chromosome suspensions. We repeatedly checked the purity of the flow-sorted fractions by microscopic observation of 3DS chromosomes sorted on a glass slide and subjected to FISH with probes for GAA microsatellite and *Afa* repeats. On average, the sorted fractions consisted of 91% chromosomes 3AS and 86% chromosomes 3DS. Contaminating particles were arms and chromatids of other chromosomes without an apparent prevalence of specific types. Pulsed field gel electrophoresis showed that the DNA of both sorted chromosome arms was intact, indicating its usefulness for BAC library construction.

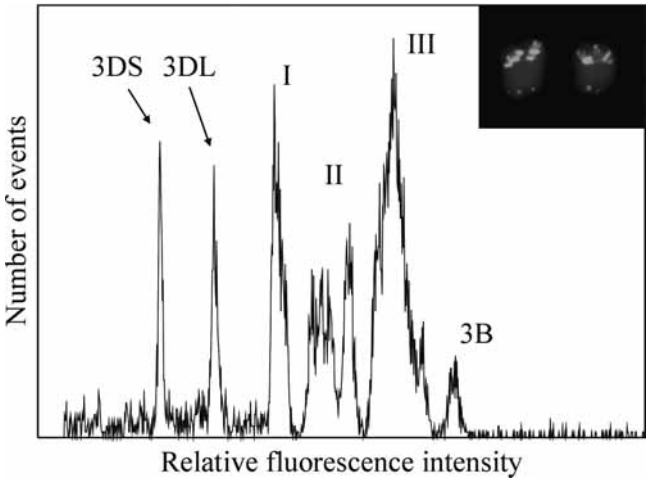

FIGURE 2 - Histogram of relative fluorescence intensity ("flow karyotype") obtained after flow cytometric analysis of DAPI-stained suspension of chromosomes prepared from 3DS ditelosomic line of wheat cultivar Chinese Spring. The flow karyotype consists of three composite peaks (I, II, and III) representing specific groups of chromosomes, a peak representing chromosome 3B and peaks representing short (3DS) and large (3DL) chromosome arms of 3D. Examples of flow-sorted chromosomes 3DS are shown in the insert after FISH with a probe for telomere repeats (red signals) and GAA microsatellite (green signals). The chromosomes were counterstained with DAPI (blue color).

DNA of sorted chromosomes was partially digested with *Hind*III and used to prepare 3AS (library code TaaCsp3AShA) and 3DS (library code TaaCsp3DShA) BAC libraries. The complete 3AS BAC library consists of three sub-libraries created from three independent ligation reactions using different sized classes of DNA as C2: 75 – 100 kb, C3: 100 – 150 kb and C4: 150 – 200 kb. We estimated the average insert size of the library after the analysis of 200 BAC clones selected randomly from the three sub-libraries (Figure 5). Differences in average insert size between three sub-libraries were not observed. The complete 3AS-specific BAC library comprises 55,296 clones ordered in 144 384-well plates with an average insert size of 80 kb (C2, C3 and C4 sub-libraries were made of 7,296, 46,080 and 768 clones, respectively). The smallest BAC clone has 10 kb insert while the largest one has 215 kb. Almost a one third of the 3AS library (30.8%) has inserts smaller than 50 kb, while 36% of clones carry inserts larger than 100 kb (Figure 3). Considering the molecular size of 3AS = 360 Mbp, the library was estimated to cover the 3AS chromosome arm 12.0-fold.

Two rounds of PFGE size selection were performed for the construction of 3DS specific BAC library (library code TaaCsp3DShA). Two regions were excised and electroeluted from the second gel and size-selected DNA was ligated into BAC vector. Based on analysis of 200 randomly

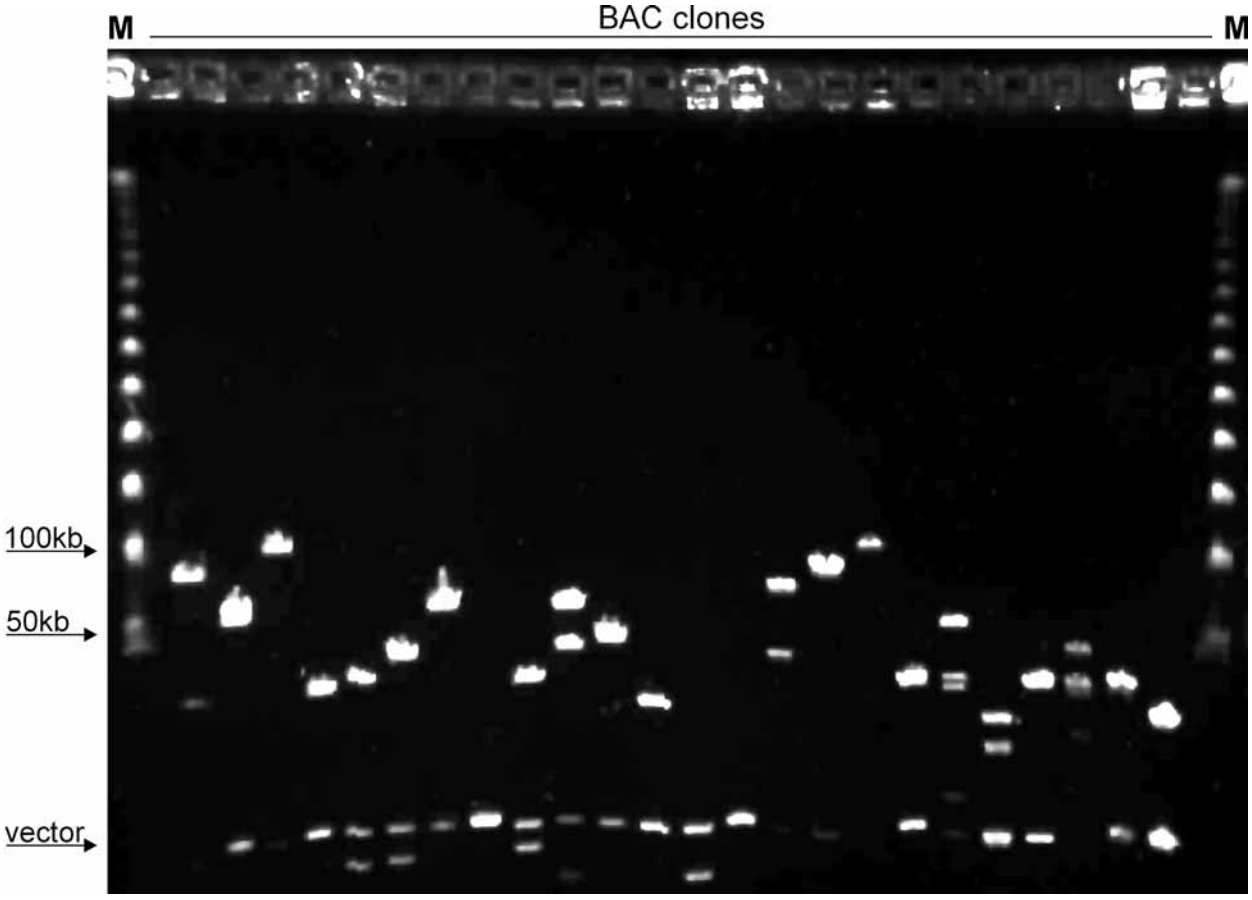

FIGURE 3 - Insert-size analysis of 24 randomly selected BAC clones from the 3AS-specific 3AS library. BAC DNA was digested with NotI enzyme to release the insert, separated by pulsed field gel electrophoresis (PFGE) and stained with ethidium bromide. Lanes M contain λ PFGE marker. The 7.5-kb band represents the BAC vector.

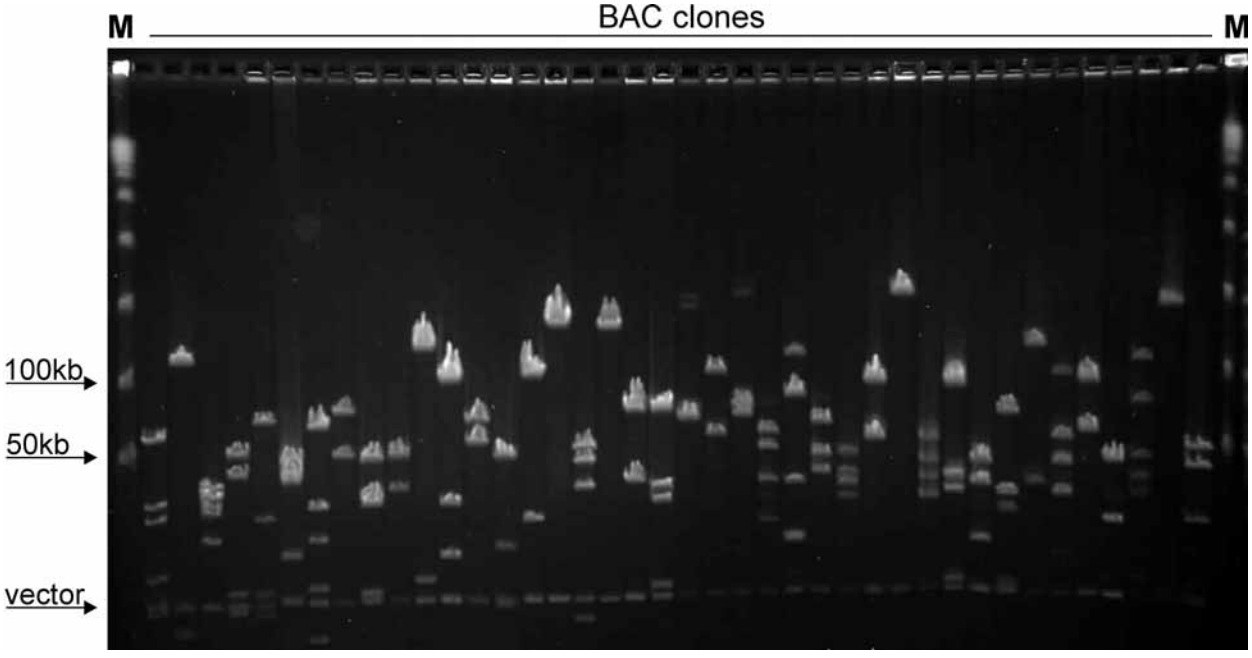

FIGURE 4 - Insert-size analysis of 40 randomly selected BAC clones from the 3DS-specific BAC library. BAC DNA was digested with NotI enzyme to release the insert, separated by pulsed field gel electrophoresis (PFGE) and stained with ethidium bromide. Lanes M contain λ PFGE marker. The 7.5-kb band represents the BAC vector.

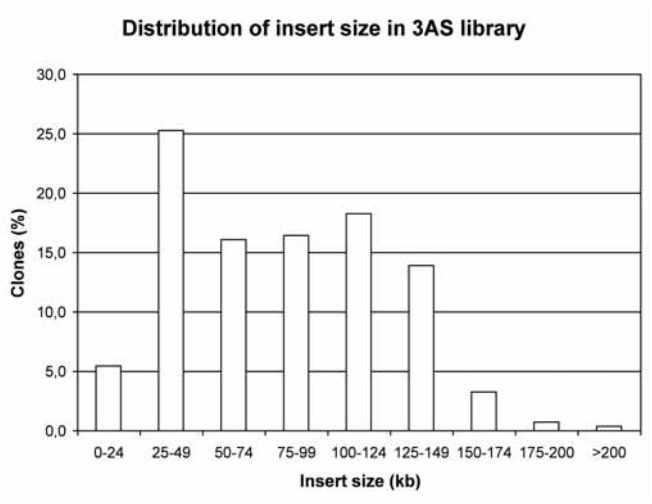

FIGURE 5 - Insert size distribution in the 3AS-specific BAC library. 200 random clones from the 3AS BAC library (TaaCsp3AShA) were isolated and sized using PFGE.

picked clones (Figure 6), it was found that the region labeled M (= middle, 150-200 kb) resulted in clones with 129 kb average insert size. Unfortunately, 23% of analyzed clones did not carry inserts and thus BAC clones derived from this region were not included into the library. Only clones derived from region B (= bottom, 100-150 kb) were collected. The complete 3DS-specific BAC library comprises 36,864 clones ordered in 96 384-well plates. The average insert size was estimated to 110 kb. The smallest insert found had 30 kb while the largest was 160 kb. Only a 4% of the 3DS library carries inserts smaller than 50 kb, while more than 82.7% inserts are larger than 100 kb (Figure 4). The percentage of

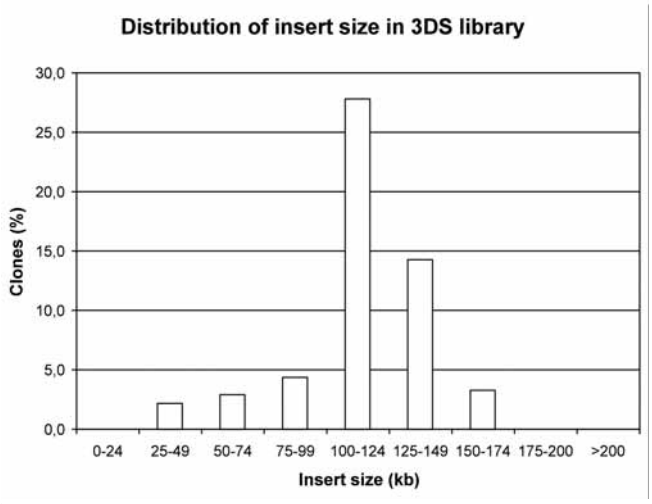

FIGURE 6 - Insert size distribution in the 3DS-specific BAC library. 200 random clones from the 3DS BAC library (TaaCsp3DShA) were isolated and sized using PFGE.

empty clones was 4.7%. Considering the molecular size of 3DS = 321 Mbp, the library was estimated to cover the 3DS chromosome arm 11-fold.

Distribution of average insert size from clones specific for 3AS (old protocol) and distribution of average insert sizes from 3DS specific BAC library (new protocol) are shown on Figure 5 and 6, respectively.

In this paper we report an important step forward towards the completion of BAC resources for the homoeologous chromosome group 3 of wheat. Until now, BAC libraries were create from wheat chromosomes 1D, 4D and 6D (JANDA *et al.*, 2004), 3B (ŠAFÁŘ *et al.*, 2004) and 1BS (JANDA *et al.*, 2006). Our results also confirm that it is realistic to plan the production of BAC libraries for all wheat chromosomes and chromosome arms. This is made possible by the availability of a complete series of telosomic lines in wheat (SEARS, 1954) and by a possibility to purify wheat telosomes by flow cytometry sorting (KUBALÁKOVÁ *et al.*, 2002).

The most obvious advantage of the chromosome-based BAC libraries is their specificity as it avoids most of the difficulties due to homoeology in physical mapping and positional cloning in a polyploid species. The contamination of libraries with other chromosomes depends on the accuracy of sorting. Although it is has been possible to sort wheat chromosomes with purities up to 97% (KUBALÁKOVÁ *et al.*, 2002), typically, purities in large-scale chromosome sorting experiments have ranged from 88% to 91% (JANDA *et al.*, 2004, 2006, ŠAFÁŘ *et al.*, 2004). The identity of sorted particles was confirmed by FISH (Fluorescence In Situ Hybridization) or PRINS (PRimed IN Situ DNA labeling) using probes and primers that provide chromosome-specific fluorescent labeling (VRÁNA *et al.*, 2000, ŠAFÁŘ *et al.*, 2004) as well as by screening of the BAC libraries with chromosome-specific markers (Janda et al. 2004, 2006, ŠAFÁŘ *et al.* 2004). The results showed that chromosome-specific BAC libraries contained about 10% of contaminating clones. However, the results of a pilot project on development of physical map of chromosome 3B indicate that this low level of contamination has no effect on the accuracy of the results and therefore does not affect the value of chromosome-specific BAC libraries. A minimal tiling path (MTP) of chro-

mosome 3B has already been established and can be used for structural and functional studies on chromosome 3B (PAUX *et al.*, 2008).

Chromosome (arm)-specific BAC libraries are a useful source of genome specific markers for marker assisted selection. This is highly relevant for polyploid species such as wheat in which the presence of homoeologous sequences hampers the efficient development of genome specific markers. It has been shown recently that BAC end sequences (BES) from chromosome specific BAC libraries can be used very efficiently to develop a large number of genome specific markers for genetic and physical mapping in wheat (PAUX *et al.*, 2006, BARTOŠ *et al.*, 2008) by using the junctions between repetitive elements. The main advantage of these Insertion Site Based Polymorphism (ISBP) markers is that they are very specific, polymorphic, and distributed homogeneously along the chromosomes in contrast to EST or SSR markers that can show some bias in genomic distribution.

In addition to simplifying the physical mapping by targeting specific chromosomes, the use of chromosome BAC libraries offers important logistical advantages. With a number of clones ranging from 4×10^4 to 10^5 (ordered in 96 to 260 $\times$ 384-well plates), the libraries maintenance (replication, pooling) and screening is easier and cheaper than genomic BAC libraries that comprise 10 – 30 times more clones (1000 to >2600 $\times$ 384-well plates; ALLOUIS *et al.*, 2003; LING and CHEN, 2005; NILMALGODA *et al.*, 2003; RATNAYAKA *et al.*, 2005). Finally, the possibility of approaching the hexaploid wheat genome one chromosome at a time allows the establishment of a strategy for physical mapping that is based on international collaborations in which individual laboratories develop physical maps of specific chromosomes and chromosome arms for a reasonable cost and labor investment with progress in each laboratory independent of the others.

An apparent limitation of the chromosome BAC libraries is the insert size, which is at the lower range of published genomic BAC libraries (ALLOUIS *et al.*, 2003; LING and CHEN, 2005). Although the chromosome BAC libraries contain clones with inserts up to 200kb, the presence of clones with inserts shorter than 50 kb downgrade the average insert size (JANDA *et al.*, 2006). Due to the small amount of starting DNA, the original protocol for construction of chromosome BAC libraries involved only one DNA size selection step (ŠAFÁŘ *et al.*, 2004). In this work we compare the effectiveness of the single step selection method versus the double selection procedure, The single selection method was used to construct 3AS-specific BAC library and the parameters of the library (Figure 3 and 5) were similar to our previous experiments as 31% clones have inserts smaller than 50 kb. However, the clones with inserts bellow 50 kb complicate fingerprint analysis and building contigs during physical map construction as they are classified as non-informative. In order to reduce the presence of clones with shorter inserts, we improved the protocol so that it is possible to make two size selection steps even with the small amounts of starting DNA. The improved protocol was used to construct BAC library from 3DS. When compared with the first generation chromosome BAC libraries, the 3DS BAC library is characterized by greatly improved average insert size and absence of clones with shorter inserts (Figure 4). More than 96% of clones carry informative inserts for fingerprinting analysis.

In conclusion, our work makes available new subgenomic BAC resources representing only few per cent of the whole bread wheat genome and allows to a further step towards homoeologous chromosome group 3 physical mapping. For the first time we present the results obtained with an improved protocol for BAC library construction from sorted chromosomes. The second generation BAC libraries thus obtained shows an average insert size greatly improved and libraries are free of short insert clones. We envisage that the use of the 3AS- and 3DS-specific BAC libraries will accelerate the development of sequence-ready physical contig maps and gene cloning, as well as comparative analyses aiming at revealing the genome changes accompanying the evolution of homoeologous chromosome group 3.

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