

Development of Chromosome-Specific BAC Resources for Genomics of Bread Wheat

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Abstract

The large bread wheat genome (1C ~ 17 Gbp) contains a preponderance of repetitive DNA and the species is polyploid. These characteristics together serve to hamper the molecular analysis of the wheat genome. Its complexity can, however, be reduced by using flow cytometry to isolate individual chromosomes, and these can be exploited to construct chromosome-specific BAC libraries. Such libraries simplify the task of physical map construction, positional cloning and the targeted development of genetic markers. Rapid improvements in the efficiency and cost of DNA sequencing provide an opportunity to contemplate sequencing the wheat genome by preparing sequence-ready physical maps for each chromosome or chromosome arm in turn. The quality of the chromosome-specific libraries depends on their chromosome coverage and the mean insert size. First-generation libraries suffered from a relatively low mean insert size, but improvements to the protocol have generated a second wave of libraries with a significantly increased mean insert size and better chromosome coverage. Each chromo-

some (arm)-specific library is composed of a manageable number of clones, and so represents a practical tool in the area of wheat genomics.

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Hexaploid wheat (*Triticum aestivum* L., $2n = 42$) provides a source of staple food for 35% of the world's population, an importance challenged only by rice. Annual global wheat consumption exceeds 600 Mt (<http://www.fao.org/>), equivalent to about 100 kg per capita. Wheat is cultivated over a wide range of climates and soil conditions, from as far north as the Arctic Circle to as far south as the equator. Despite its major socio-economic importance and the current challenges faced by agriculture [Wollenweber et al., 2005], investment in wheat genomics and its application to crop improvement continues to lag behind those of other leading crop species. Its particular disadvantage, compared to other crops, lies in its large genome size (1C ~ 17 Gbp), brought about by the presence of so much repetitive DNA [Paux et al., 2006] and its allohexaploid status. These properties complicate the process of whole-genome sequencing. Genetic mapping in wheat is hampered both by a very uneven spatial distribution of recombination [Saintenac et al., 2009] and a

rather low level of polymorphism [Suenaga et al., 2005], making the prospect of positional gene cloning a highly challenging one. To date, fewer than a dozen genes have been isolated from wheat in this way [Devos et al., 2009; Krattinger et al., 2009]. Thus significant improvements in wheat genome technology are needed to accelerate breeding via modern genetic methods.

A variety of genetic resources and technical platforms are available to develop marker-assisted selection assays, identify and isolate genes underlying important traits and understand the evolution and function of the polyploid wheat genome. The former include an unrivalled range of cytogenetic stocks [Sears, 1954; Sears and Sears, 1978; Endo and Gill, 1996] and many mapping populations (<http://wheat.pw.usda.gov/>). Among the latter are various molecular marker types [Somers, 2003; Akbari et al., 2006; Paux et al., 2006; Banks et al., 2009; <http://wheat.pw.usda.gov/>], genetic maps [reviewed in Gupta et al., 2008; <http://wheat.pw.usda.gov/>], radiation hybrid maps [Kalavacharla et al., 2006; Paux et al., 2008], cDNA libraries and ESTs [Ogihara et al., 2004; <http://wheat.pw.usda.gov/>; <http://www.ncbi.nlm.nih.gov/>], genomic DNA libraries and DNA arrays [Akbari et al., 2006; Banks et al., 2009; Luo et al., 2009]. However, the ultimate resource remains the complete genome sequence. Although this has been seen as unattainable in the past, rapid developments in DNA sequencing technology have led to a scenario where gigabases of sequence can be derived within just a few days [Mardis, 2008]. What still remains an insurmountable problem is the assembly of many millions of sequence reads into a coherent contig, largely because of the high representation of dispersed repeats and the complication introduced by polyploidy. Current genome sequencing strategy relies on the shotgun approach, which requires the assembly of many small fragments to reconstitute the genome. This has replaced the now outmoded approach which was based on the construction of a series of overlapping BAC clones, known as the minimum tiling path (MTP), but a return to this strategy may well hold the key to successful wheat genome sequencing. An MTP also simplifies both the process of marker development, since the location of the target locus is defined by a single clone (the size of which is typically ~100 kb), and that of positional cloning. The current review focuses on a chromosome-based strategy suitable for the development of a physical map of wheat, and describes the construction of a set of chromosome-specific bacterial artificial chromosome (BAC) libraries, which represent a key component in this strategy.

Clone-Based Physical Maps

The construction of a clone-based physical map requires the identification of groups of clone inserts which overlap with one another. These overlaps are assembled into a continuous sequence (a contig), which ideally can be extended to cover the whole genome. The recognition of overlaps is achieved by comparing restriction fragment profiles or 'fingerprinting' [Coulson et al., 1986; Ding et al., 2001]. A widely-used fingerprinting platform is the SNaPshot HICF (high information content fingerprinting) technique described by Luo et al. [2003], in which the restriction profiles derived by digestion with 5 enzymes are separated by capillary electrophoresis. The advantages of this platform relate both to its resolving power and its throughput efficiency. The FPC fingerprint editing software then assembles the clones into contigs [Soderlund et al., 2000]. A disadvantage of this approach is that it cannot provide DNA sequence information, which would help to locate each contig on a genetic map. The integration of the physical with the genetic map is required before it can be used for positional cloning and sequence assembly. This step is conventionally achieved by testing contigs with appropriate PCR- or hybridization-based assays. However, the dramatic fall in the cost of DNA sequencing now offers a means of assembling the physical maps on the basis of restriction fragment end sequences, rather than relying on fragment size. However, as yet, this approach has only been validated using diploid species with genome size orders of magnitude smaller than that of wheat [van Schriek et al., 2009].

Large-insert genomic libraries are the basis of clone-based physical maps. This was first made possible in yeast, by the development of yeast artificial chromosome (YAC) libraries, in which insert sizes of up to 1 Mb were feasible [Burke et al., 1987]. While YACs proved useful for the physical mapping of mammalian genomes [Chumakov et al., 1992], it later became clear that insert stability was problematical. A further disadvantage of YACs was the technical difficulty associated with DNA purification. Thus YAC libraries have been gradually abandoned in favor of either BAC [Shizuya et al., 1992] or P1-derived artificial chromosome (PAC) libraries [Ioannou et al., 1996]. Of these, BACs are generally preferred for physical mapping and sequencing [Zhang and Wu, 2001]. The insert size of BACs (100–200 kb) is considerably less than that of YACs, the libraries are easier to maintain and reproduce, show only a low level of chimerism, and are amenable to screening either by PCR or hybridization methods. Moreover, the inserts can be readily isolated by simple plasmid extraction procedures.

Large-Insert Wheat Libraries

A number of bread wheat BAC libraries have been constructed [Allouis et al., 2003; Nilmalgoda et al., 2003; Ling and Chen, 2005; Ratnayaka et al., 2005; Shen et al., 2005]. The number of clones within them ranges from ~400,000 to 1,200,000, representing 3.1–9.3 genome equivalents. (Note that a 15× genome coverage would require ~2,500,000 clones of average size 100 kb.) While fingerprinting such large numbers of clones is technically feasible using HICF, existing computational techniques may not allow their reliable assembly into contigs which would faithfully represent the individual chromosomes. Recent results gained with fingerprinting BAC clones derived from the 3 homoeologous chromosomes 3A, 3B and 3D do, however, indicate that this is feasible [Luo et al., 2010]. Construction, analysis and maintenance of a BAC library of this size would be an expensive and cumbersome undertaking. Although non-gridded BAC libraries [Liu et al., 2000; Ma et al., 2000] may be useful for some applications, these are unsuitable for physical map construction, which requires individual clones. Thus various strategies have been elaborated to avoid the need to generate a complete BAC library in wheat. The problem of polyploidy was neatly sidestepped by targeting the diploid progenitors [Feuillet et al., 2003; Huang et al., 2003; Yahiaoui et al., 2004], so that libraries are now available for the D genome progenitor *Aegilops tauschii* [Moulet et al., 1999; Akhunov et al., 2005], the A genome diploids *T. monococcum* [Lijavetzky et al., 1999], *T. boeoticum* [Chen et al., 2002] and *T. urartu* [Akhunov et al., 2005], the S genome type species (ancestor of the B genome) *Ae. speltoides* [Akhunov et al., 2005], and the AB tetraploid durum wheat *T. turgidum* ssp. *durum* [Cenci et al., 2003]. While these libraries are more manageable than a hexaploid-based one, they remain clumsy, as the genome size of each of the diploids is twice the size of that of maize, so that the generation of physical maps still represents a major technical challenge [Devos et al., 2009].

The development of a physical map of *Ae. tauschii* is the primary goal of a current NSF project (grant 0701916). A complication of relying on diploid wheat as a surrogate for the hexaploid is that their genomes differ from one another by a number of ‘evolutionary’ translocations and other DNA rearrangements [Wicker et al., 2003; Chantret et al., 2005; Feldman and Levy, 2005]. As a result, the genome does not correspond exactly at either the structural or the functional level to the sum of its 3 component ancestral diploid genomes. A rather different way of reducing the complexity of the wheat BAC library and to

simplify the construction of the physical map relies on the possibility of isolating single chromosomes or chromosome arms [Doležel et al., 2007]. Each chromosome represents only a few percent of the total genome. The approach avoids the complication of interference between homoeologues, and the size of the library is reduced by an order of magnitude or so. The approach has been pioneered by the successful production of a physical map of chromosome 3B, and has been formally adopted as the platform for whole-genome sequencing in wheat (<http://www.wheatgenome.org/>). The key to this strategy is that the DNA of isolated chromosomes is suitable for the construction of a BAC library.

Flow Cytometry of Wheat Chromosomes

The basis for separating individual chromosomes from one another is flow cytometry, a technology in which fluorescently labeled objects (in this case mitotic chromosomes) are moved past a sensor in a fast moving stream. Flow cytometry can process ~1,000 intact mitotic plant chromosomes per second. These chromosomes are typically labeled with the DNA-specific dye DAPI, and the analysis generates a histogram of relative fluorescence intensity (called the ‘flow karyotype’). In the ideal scenario, each chromosome is represented by a single peak on a flow karyotype. A typical flow cytometer and sorter can simultaneously sort 2 different populations of particles (chromosomes). With the analysis rate of 1,000 particles/second, one chromosome type can be sorted at rates of 15–30/second (the lower than theoretical rate is due to the presence of debris particles in the sample).

A viable procedure for preparing intact wheat mitotic chromosomes was developed by Vrána et al. [2000]. The chromosomes are released into solution by the mechanical homogenization of formaldehyde-fixed root tips enriched for mitotically dividing cells stopped at metaphase [Doležel et al., 1992]. Approximately 0.5×10^6 such chromosomes can be isolated from 30 root tips [Vrána et al., 2000]. The flow karyotype of bread wheat comprises 3 large peaks, each representing a mixture of chromosomes of similar size, and a minor peak composed largely of chromosome 3B (fig. 1A). Thus, in the standard flow karyotype, only one out of the 21 chromosomes can be sorted [Vrána et al., 2000]. Access to the other 20 chromosomes was made possible by the well-documented ability of hexaploid wheat to tolerate aneuploidy, which has allowed a wide spectrum of cytogenetic stocks to be

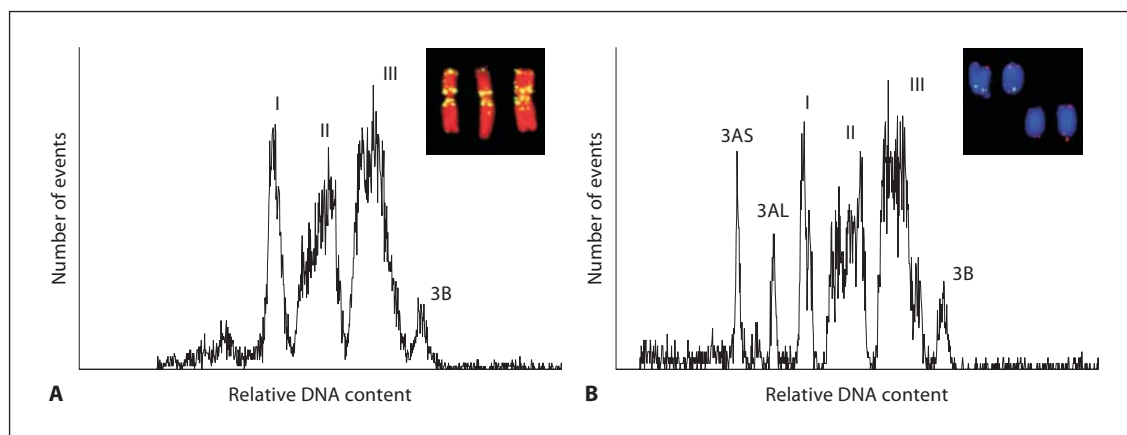


Fig. 1. Chromosome analysis and sorting in wheat. **A** Histogram of relative fluorescence intensity (flow karyotype) of the common wheat cultivar 'Chinese Spring' obtained after flow cytometric analysis of DAPI-stained chromosome suspension. The karyotype consists of 3 composite peaks (I, II and III) representing groups of chromosomes and a clearly discriminated peak representing chromosome 3B. The inset shows examples of flow-sorted chromosome 3B after FISH with a probe for GAA microsatellite (yellow signals). The chromosomes were counterstained with

DAPI (red pseudocolor). **B** Flow karyotype obtained after flow cytometry analysis of a DAPI-stained suspension of chromosomes prepared from double ditelosomic line 3A of wheat cultivar 'Chinese Spring'. Two peaks representing short (3AS) and long (3AL) chromosome arms, respectively, are visible. The inset shows examples of flow-sorted 3AS (top left) and 3AL (bottom right) after FISH with a probe for telomere repeats (red signals) and GAA microsatellite (green signals). The chromosomes were counterstained with DAPI (blue color).

bred [Sears, 1954; Islam et al., 1981; Endo and Gill, 1996]. In particular, plants in which one (or both) of the arms of a particular chromosome are inherited stably have facilitated the sorting of chromosomes other than 3B, since the individual arms of most of the wheat chromosomes are smaller than the smallest intact chromosome [Kubaláková et al., 2002]. The flow karyotypes of such lines include a peak(s) not present in the standard flow karyotype (fig. 1B). In principle, this opens the door to dividing the 17-Gbp genome into separate fractions of 224–580 Mb, each representing only 1.3–3.4% of the whole genome, a significant reduction in complexity.

Construction of Chromosome-Specific BAC Libraries

The DNA used for BAC library construction must be as unsheared as possible. Šimková et al. [2003] demonstrated that a very short exposure of DAPI-stained chromosomes to strong UV light during flow cytometric analysis and sorting caused no detectable damage to the chromosomal DNA. Critical steps in the BAC library construction protocol are the preparation of high molecular weight (HMW) DNA, its partial digestion, size selection using pulsed-field gel electrophoresis (PFGE) to select DNA fragments of appropriate size (100–300 kb), the

release of DNA from the gel, its ligation into a BAC vector and the transformation of competent cells [Zhang et al., 1996; Peterson et al., 2000]. The overall process conventionally sets out with 10–20 μg of DNA, a large amount in the context of chromosome sorting, where 1 μg of DNA is equivalent to $\sim 1,300,000$ chromosomes. Šimková et al. [2003] developed a novel means of HMW DNA extraction from flow-sorted chromosomes. This involved the embedding of batches of 100,000–200,000 chromosomes/chromosome arms in low-melting-point agarose plugs, which were treated with proteinase K to prevent damage by the nucleases released after cell lysis. The purity of each sorted fraction can be monitored by in situ hybridization [Doležel et al., 2004] to detect and exclude chromosome mixtures from being included in the library. A typical daily yield of sorted chromosomes is $\sim 200,000$, so a 20- μg harvest implies sorting for at least 100 days. As this period was considered unrealistic, a lower target of 5 μg was set (table 1). The successful construction of the chromosome 3B-specific BAC library [Šafář et al., 2004] demonstrated the technical feasibility of the approach.

Chromosome-specific BAC libraries have been successfully constructed based on either in-house [Janda et al., 2004, 2006; Šafář et al., 2004] or commercially available vectors [Šafář et al., 2007; Šimková et al., 2008]. It is

Table 1. Molecular size of wheat chromosomes and chromosome arms

Chromosome ^a	Size Mb	Chrs/5 μ g DNA	BACs/15 \times ^b	Short arm	Size Mb	Chrs/5 μ g DNA	BACs/15 \times ^b	Long arm	Size Mb	Chrs/5 μ g DNA	BACs/15 \times ^b
1A	799			1AS	275	8,865,248	45,833	1AL	523	4,672,897	87,167
2A	899			2AS	391	6,250,000	65,167	2AL	508	4,807,692	84,667
3A	827			3AS	360	6,793,478	60,000	3AL	468	5,230,125	78,000
4A	856			4AS	317	7,716,049	52,833	4AL	539	4,537,205	89,833
5A	827			5AS	295	8,278,145	49,167	5AL	532	4,595,588	88,667
6A	705			6AS	336	7,288,629	56,000	6AL	369	6,613,756	61,500
7A	813			7AS	407	6,009,615	67,833	7AL	407	6,009,615	67,833
1B	849			1BS	314	7,788,161	52,333	1BL	535	4,570,383	89,167
2B	928			2BS	422	5,800,464	70,333	2BL	506	4,826,254	84,333
3B	993	2,463,054	165,500	3BS	432	5,668,934	72,000	3BL	561	4,355,400	93,500
4B	820			4BS	319	6,265,664	53,166	4BL	430	5,694,760	71,667
5B	871			5BS	290	8,417,508	48,333	5BL	580	4,215,851	96,667
6B	914			6BS	415	5,882,352	69,167	6BL	498	4,901,960	83,000
7B	889			7BS	360	6,793,478	60,000	7BL	540	4,528,985	90,000
1D	604			1DS	224	10,917,030	37,333	1DL	381	6,426,735	63,500
2D	727			2DS	316	7,739,938	52,667	2DL	411	5,952,380	68,500
3D	770			3DS	321	7,621,951	53,500	3DL	449	5,446,623	74,833
4D	648			4DS	231	10,593,220	38,500	4DL	416	5,868,544	69,333
5D	748			5DS	258	9,469,696	43,000	5DL	490	4,990,019	81,667
6D	712			6DS	324	7,552,870	54,000	6DL	389	6,297,229	64,833
7D	727			7DS	381	6,426,735	63,500	7DL	346	7,062,146	57,666
Genome	16,937	–	2,540,550 ^c	–	–	–	–	–	–	–	–

^a Only the 3B chromosome can be sorted as a whole chromosome. ^b Assuming 100 kb mean insert size, 90% purity in sorted fraction and 15 \times coverage. ^c Assuming 100 kb mean insert size and 15 \times genome coverage.

important to confirm the quality of the cohesive ends and that dephosphorylation is complete. Most BAC vectors contain *Hind*III, *Bam*HI and *Eco*RI cloning sites, and the general recommendation is to use a spread of enzymes to maximize genome coverage [Tao et al., 2001]. In our experience, *Bam*HI and *Eco*RI are less efficient than *Hind*III, perhaps because both are more prone to star activity under suboptimal reaction conditions – this can result in a reduction of the quality of cohesive ends both in the vector and the insert, and thus compromise ligation efficiency. Since the amount of available chromosome-specific DNA was limited, the chromosome-specific BAC libraries we have constructed were exclusively based on *Hind*III digestion (<http://olomouc.ueb.cas.cz/dna-libraries/cereals>). This does not appear to have created a significant bias against particular genomic regions, as the chromosome 3B physical map covers 99% of the chromosome [Paux et al., 2008 and unpubl. data].

Following partial digestion and prior to ligation, the DNA needs to be size-selected by PFGE. Standard BAC library construction protocols include 2 or 3 rounds of size selection [Peterson et al., 2000; Chalhoub et al., 2004],

but again due to the limited amount of DNA available, we initially restricted this to a single round [Janda et al., 2004, 2006; Šafář et al., 2004, 2007; Šimková et al., 2008], and decreased the concentration of TBE buffer in the gel to both improve separation and increase transformation efficiency [Chalhoub et al., 2004]. Other steps which needed to be adjusted to minimize DNA loss and maximize cloning efficiency were the rate of DNA recovery, the constitution of the ligation reaction, and the effectiveness of the post-ligation desalting. We used β -agarase (gelase) to recover the DNA and dialysis tubes for its desalting. The heat treatment conventionally applied in the agarase protocol tends to damage DNA, and electroelution [Peterson et al., 2000] appeared to be less injurious. To minimize DNA shearing, the components of the ligation reaction were mixed by gentle inversion only and incubated overnight at 16°C. Desalting the ligated DNA prior to transformation is most effectively achieved using Millipore nitrocellulose filters [Peterson et al., 2000] or by a dialysis method based on 1% aqueous agarose/100 mM glucose [Chalhoub et al., 2004]. Finally, the choice of an *E. coli* cell line can have a major influence on cloning efficiency.

First-Generation Chromosome-Specific BAC Libraries

A chromosome 3B-specific library [Šafář et al., 2004] was the first to be produced, given that this chromosome is separated in the standard flow karyotype, being the largest chromosome (993 Mb) of the wheat complement. The library was created from 1.8×10^6 sorted chromosomes (3.65 μ g DNA), comprised $\sim 68,000$ clones of a mean insert size of 103 kb, and represented a $6.2\times$ coverage of the chromosome (table 2). The same protocol was then used for the construction of a multi-chromosome-specific library from 10^7 chromosomes 1D, 4D and 6D present in peak I of the standard flow karyotype (fig. 1). This library comprised $\sim 87,000$ clones, with an average insert size of 85 kb, equivalent to a $3.4\times$ coverage (table 2) [Janda et al., 2004]. Because the limited amount of DNA enabled only 1 size-selection step, the size window for cloning was reduced from the normal 100–300 kb to 50–250 kb, which resulted in the rather low mean insert size. For these 2 libraries, PFGE was performed in 1% agarose at 6 V/cm, 12°C for 14 h with a 1–40 s switching interval and an angle of 120°. In an effort to improve the separation of large DNA fragments and to remove small trapped DNA molecules, these conditions were modified by lengthening the electrophoresis to 12 h with a 1–40 s switching interval plus 6 h with a 2.5–5.5 s switching interval. Although preliminary experiments indicated an improvement in size selection, the subsequent libraries of chromosome arms 1BS [Janda et al., 2006] and 3AS [Šafář et al., 2007] had a mean insert size of <100 kb (table 2).

Second-Generation Chromosome-Specific BAC Libraries

The necessity of increasing the average insert size required the introduction of a second DNA size selection step. Since this inevitably implied additional loss of DNA, we needed to improve cloning efficiency. This was achieved by a stringent selection of vector and host. We refer to the libraries produced with this protocol as second-generation libraries; 85–95% of the clones in these libraries have an insert >100 kb (fig. 2), the proportion of clones carrying inserts <50 kb is under 2–3%, and that of insertless clones only 1–2%. As a result, >95% of the clones are suitable for fingerprinting. This protocol was employed to construct BAC libraries specific for chromosome arms 1AS, 1AL, 3AS, 3AL, 3DS, 3DL, 7DS and 7DL (table 2). Whereas the above libraries used *E. coli* DH10B

(Invitrogen, Carlsbad, Calif., USA) as a host, the following batch (1AL, 1BS, 1BL, 3AL, 4AL, 5AS and 5AL; table 2) used MegaX DH10B (Invitrogen), which offer an improved cloning efficiency and carry resistance to bacteriophages T1 and T5 (which reduces the risk of colony loss due to phage infection). The improved cloning efficiency and larger mean insert size meant that the same amount of chromosome-specific DNA should generate enough clones to give $15\times$ coverage.

Customized Chromosome-Specific BAC Libraries

Various cultivars of bread wheat carry genes underlying important traits of interest. However, for the moment, most of the chromosome-specific BAC libraries to be constructed will be derived from cv. Chinese Spring. A solution allowed by the improvement in cloning efficiency may be to construct customized libraries based on a smaller population of chromosomes with less stringent DNA size selection and both fewer clones and smaller inserts than the reference BAC libraries. This would simplify and cheapen their construction. The general feasibility of this approach has been confirmed by the experience with constructing a cv. Hope chromosome 3B BAC library using only 10^6 copies of the chromosome [Šimková et al., unpubl. data]. The library has been used to support map-based cloning of the *Sr2* gene (see below). Clearly, this approach is feasible only for chromosome 3B, and a group of 1D, 4D, and 6D chromosomes that can be sorted from any cultivar of wheat (fig. 1A). In order to sort other chromosomes, telosomic lines would have to be developed from specific cultivars. Their preparation and production of enough seed may take about 2 years [A. Lukaszewski, pers. commun.].

Wheat BAC Resources Platform

The International Wheat Genome Sequencing Consortium (IWGSC, <http://www.wheatgenome.org>) aims to construct a physical map for each cv. Chinese Spring chromosome, to provide a reference for the wheat sequencing community. The plan anticipates a BAC library from each of 34 chromosome arms, one from chromosome 3B and one from a pool of chromosomes 1D/4D/6D. Their construction presents a major challenge both in terms of logistics and quality control. A production line has been established at the Laboratory of Molecular Cytogenetics and Cytometry (<http://olomouc.ueb.cas.cz/>)

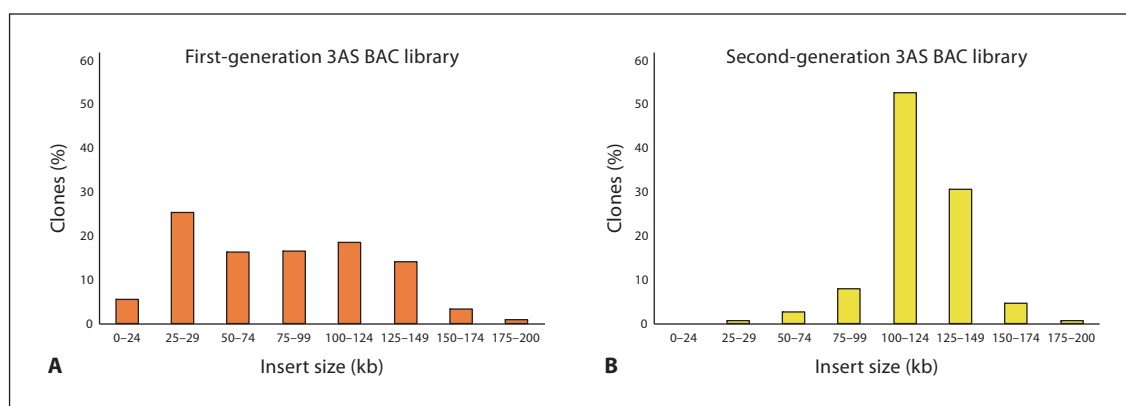


Fig. 2. Insert-size distribution in the first- and the second-generation chromosome-specific BAC libraries. **A** 3AS BAC library TaaCsp3AShA represents the first-generation BAC libraries, characterized by a lower average insert size and a large proportion of clones with inserts below 50 kb. **B** 3AS BAC library TaaCsp3A-ShB represents the second-generation of BAC libraries, characterized by an increased insert size and majority of clones (85–95%) in the fraction above 100 kb.

Table 2. List of bread wheat (*Triticum aestivum*) chromosome-specific BAC libraries

Library code	Cultivar	Chromosome	No. of clones	Insert size, kb	Purity, %	Coverage
TaaCsp146hA ^a	Chinese Spring	1D, 4D, 6D	87,168	85	91	3.4 ×
TaaCsp146hB ^b	Chinese Spring	1D, 4D, 6D	148,224	102	91	6.9 ×
TaaCsp146hC ^c	Chinese Spring	1D, 4D, 6D	138,240	116	91	7.4 ×
TaaCsp146eA ^{b, d}	Chinese Spring	1D, 4D, 6D	26,112	110	90	1.3 ×
TaaCsp1AShA ^b	Chinese Spring	1AS	31,104	111	94	11.8 ×
TaaCsp1ALhA ^b	Chinese Spring	1AL	49,536	103	83	8.0 ×
TaaCsp1ALhB ^c	Chinese Spring	1AL	43,008	109	87	7.7 ×
TaaCsp1BShA ^c	Chinese Spring	1BS	55,296	113	81	15.7 ×
TaaPav1BShA ^a	Pavon	1BS	65,280	82	90	14.5 ×
TaaCsp1BLhA ^c	Chinese Spring	1BL	92,160	114	81	15.4 ×
TaaCsp3AShA ^a	Chinese Spring	3AS	55,296	80	89	10.9 ×
TaaCsp3AShB ^b	Chinese Spring	3AS	55,296	115	91	15.9 ×
TaaCsp3ALhA ^b	Chinese Spring	3AL	55,296	106	87	10.2 ×
TaaCsp3ALhB ^c	Chinese Spring	3AL	24,576	114	88	5.2 ×
TaaCsp3BFhA ^a	Chinese Spring	3B	67,968	103	89	6.2 ×
TaaCsp3BFhB ^b	Chinese Spring	3B	82,176	126	93	9.1 ×
TaaCsp3BFhA ^{b, d}	Chinese Spring	3B	21,120	107	93	1.9 ×
TaaHop3BFhA ^e	Hope	3B	92,160	78	85	6.0 ×
TaaCsp3DShA ^b	Chinese Spring	3DS	36,864	110	90	11.0 ×
TaaCsp3DLhA ^b	Chinese Spring	3DL	64,512	105	82	12.2 ×
TaaCsp4ALhA ^c	Chinese Spring	4AL	92,160	126	81	17.3 ×
TaaCsp5AShA ^c	Chinese Spring	5AS	46,080	120	90	16.5 ×
TaaCsp5ALhA ^c	Chinese Spring	5AL	90,240	123	88	18.3 ×
TaaCsp7DShA ^b	Chinese Spring	7DS	49,152	114	84	12.2 ×
TaaCsp7DLhA ^b	Chinese Spring	7DL	50,304	115	89	14.8 ×

^a First-generation BAC libraries (one DNA size selection step). ^b Second-generation BAC libraries (two DNA size selection steps). ^c Second-generation BAC libraries cloned in phage-resistant *E. coli* cells. ^d BAC libraries prepared using *EcoRI* restriction endonuclease (other BAC libraries were constructed using *HindIII*). ^e Customized BAC library (prepared from a specific cultivar using a small quantity of DNA).

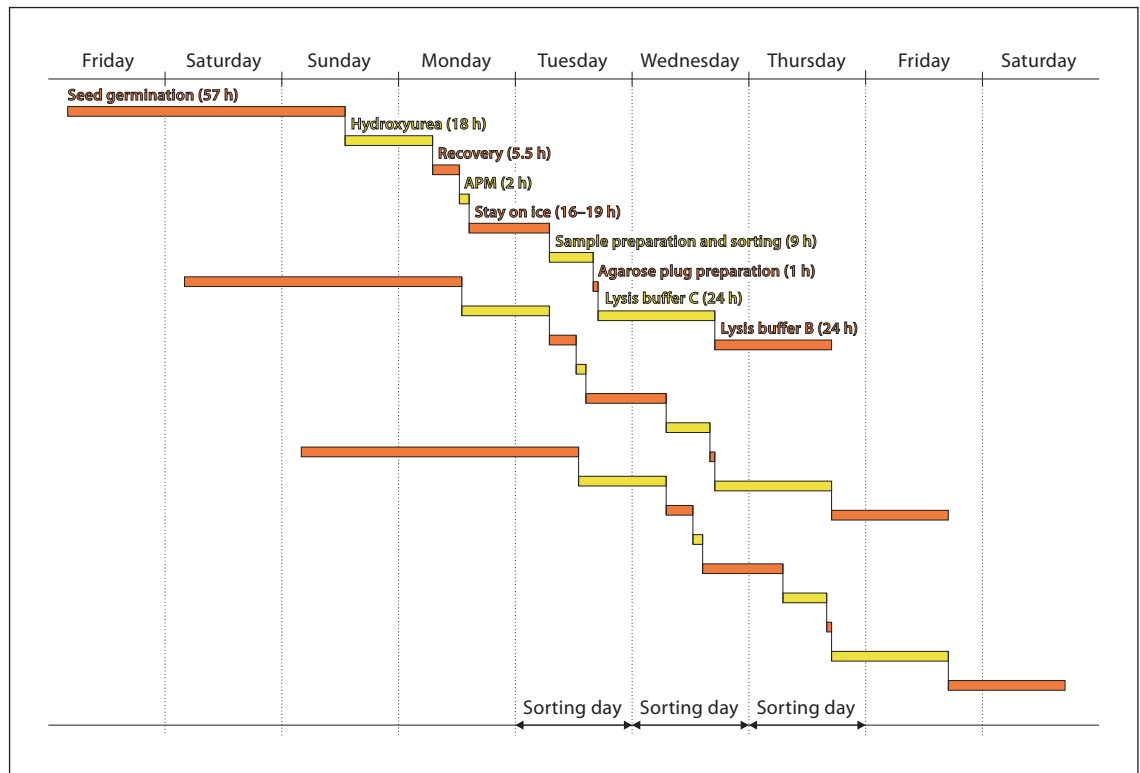


Fig. 3. Workflow of chromosome sorting and preparation of miniplugs with embedded chromosomes. The chromosomes for BAC library construction are sorted 3 days a week (Tuesday, Wednesday and Thursday). However, to do this, sample preparation needs to be started already on Friday of the previous week and the last agarose miniplugs are ready for storage only on Saturday. APM = Amiprophos methyl.

which consists of 2 sub-teams. The first of these is responsible for the flow cytometry and the second for the BAC library construction.

The flow cytometry team is equipped with 2 high-speed FACSVantage SE flow sorters (BD, San José, Calif., USA) and is responsible for the preparation, sorting and embedding of the mitotic chromosomes, including daily purity checks using *in situ* hybridization [Kubaláková et al., 2005]. Apart from the general quality of the sample and the flow cytometer parameters, purity depends on which chromosome (arm) is being sorted (the extent to which its position on the flow karyotype is close to, or overlaps that of other chromosomes, chromosome arms and chromatids). The purity achieved ranges from 81–94% (table 2), and samples which fall below this range are routinely discarded. A production pipeline is required to maintain the supply of mitotic chromosomes (fig. 3); for logistical reasons, sorting is restricted within each week to Tuesday to Thursday. With 2 flow sorters running in parallel, the average productivity is 400,000 chromo-

somes per day from 14 samples derived from 400 seeds (25 seeds are germinated per sample). Depending on the size of the sorting target (table 1), the isolation of 5 μ g DNA takes 6 to 8 weeks using 3,000–9,000 seeds. The number of agarose miniplugs required varies from 25 to 45. The storability of the miniplugs allows the schedule of the chromosome preparation sub-group to be decoupled from that of the library construction sub-group.

The BAC library construction team has 2 robotic colony pickers available: a Q-Bot (Genetix, Dorset, UK) and a GeneTAC G3 (Genomics Solutions, Huntingdon, UK). The procedure consists of partially digesting 25–45 miniplugs containing \sim 5 μ g DNA in total, and subjecting the restriction products to size selection via PFGE. Various fractions (100–125 kb, 125–150 kb and 150–225 kb) are extracted from the gel and ligated to the BAC vector. The cloning step itself requires 1 week, and generally the different size fractions are processed in serial rather than in parallel. The picking and ordering of clones derived from 1 to 3 fractions into 384-well microtiter plates, the analy-

sis of insert size and the preparation of replica plates take 3 to 4 weeks. The libraries (the master copy and 2 replicas) are maintained at -80°C . A 0.5% sample (150–300) of clones is taken to provide an estimate of mean insert size, and genome coverage is determined from the total number of clones, the average insert size, the proportion of empty clones, the extent of contamination by non-target DNA chromosomes (purity) and the size of the target chromosome or chromosome arm (table 1). The inclusion of the second size-selection step has increased the average insert size from 102 kb to 126 kb (table 2). The extent of contamination by non-target chromosomes ranges from 6–19% (mode 10%), with no evidence for any bias in the identity of the contaminating chromosomes (table 2). This level of contamination has no discernible effect on the physical maps, as the contaminating sequences tend not to integrate into a contig [Paux et al., 2008; Luo et al., unpubl. data]. As the template for the BAC library is free of cytoplasm, the libraries are essentially devoid of plastid and mitochondrial DNA [Šafář et al., 2004; Janda et al., 2006].

Applications of the Chromosome-Specific BAC Libraries

Construction of Physical Maps

Construction of physical maps is currently the major application of the libraries, which are superior to whole genome libraries because their size is an order of magnitude smaller. The number of clones lies in the range of 35,000–100,000 (table 2), ordered into 90–200 384-well plates; thus each library occupies much less -80°C space, and the work involved in replication, pooling, fingerprinting and screening is greatly reduced and less expensive. A particular advantage of these libraries is that a community-based effort to construct physical maps is straightforward to organize, as each participant can be given responsibility for a particular chromosome or chromosome arm. The value of these libraries has been shown by the development of the physical map of chromosome 3B [Paux et al., 2008], a chromosome which on its own is almost 3 times larger than the whole rice genome. This map comprised 1,036 contigs, covering $\sim 82\%$ of the chromosome. It required fingerprinting of $\sim 68,000$ clones, estimated to represent a $6.2\times$ coverage of the chromosome [Šafář et al., 2004]. The MTP consisted of 7,500 clones and a 3-dimensional DNA pool based on the MTP could be fitted into a single 96-well plate. A second *HindIII* library, giving $9.1\times$ coverage, has also been fingerprinted [Paux,

unpubl. data], and in combination with the initial one has generated a physical map covering 95–99% of the chromosome. The conclusion was that a deep ($15\times$) coverage *HindIII* library is necessary to achieve this high level of chromosome representation. The 3B physical map is in the process of being integrated with extant genetic and cytogenetic maps in preparation for a pilot chromosome sequencing effort [C. Feuillet, pers. commun.].

Progress to date has stimulated Gill et al. [2008] to fingerprint $>55,000$ BAC clones from the chromosome 3A libraries [Šafář et al., 2007], in a project funded by the USDA to construct physical maps of both arms of the chromosome. Similarly, Luo et al. [2008] have fingerprinted $\sim 37,000$ chromosome arm 3DS clones [Šafář et al., 2007] and obtained 1,360 contigs from an automated assembly [Bartoš, unpubl. data]. The EU FP7 TriticeaGenome project aims to develop physical maps of chromosome arms 1AS, 1AL, 1BS, 1BL and 3DL (<http://www.triticeaegenome.eu/pillar.php?p=1>). Efforts are under way to develop a physical map of the whole D genome, thanks to an NSF-funded project (0701916) targeted at chromosomes 1D, 4D and 6D, and to current work in our laboratory to fingerprint and contig clones from the 7DS and 4AL libraries.

Fluorescence in situ Hybridization Using BAC Clones

Cytogenetic mapping is important to support the development of physical maps, since it allows the positioning of contigs onto the chromosomes, which is especially necessary in regions where recombination is rare. Various attempts have been made to use BAC clones as fluorescence in situ hybridization (FISH) probes, but the abundance of dispersed repetitive DNA in the wheat genome has impeded the localization of BAC clones, as in most experiments the signal is spread over much, if not all, of the chromosome [Janda et al., 2004; Šafář et al., 2004; Zhang et al., 2004]. A potential strategy to overcome this problem is to use 2–3 kb ‘low-copy’ shotgun clones as FISH probes, but only about 4% of the BACs are probably analyzable in this way [Janda et al., 2006]. Thus, the approach is unlikely to be usable on a routine basis.

Targeted Development of DNA Markers

Chromosome-specific libraries and contig maps provide an ideal source of markers for saturating a genetic map in a particular region. An example has been provided by Paux et al. [2006], who identified junctions between transposable elements (TEs) and non-TE sequences among 20,000 BAC-end sequences (BESs) produced from the chromosome 3B library. These data were used to de-

velop chromosome-targeted insertion site-based polymorphism (ISBP) markers. A similar approach was taken by Bartoš et al. [2008] to identify TE junctions in chromosome arm 1RS BESs, as well as sequences carrying microsatellite motifs; the latter were used by Kofler et al. [2008] to develop 1RS-specific microsatellites. After the chromosome 3B contig spanning the *Sr2* gene was identified, McNeil et al. [2008] were able to use the MTP sequence to develop 3 molecular markers tightly linked to *Sr2*, allowing it to be tracked in breeding populations.

Genomic Organization in Localized Regions

The libraries offer an unparalleled means of analyzing localized molecular organization and evolution in the wheat genome. Paux et al. [2006] analyzed ~11 Mb of BESs from BAC clones forming the chromosome 3B MTP, and showed that these consisted of some 86% repetitive DNA, 1.2% unique (coding) sequence (equivalent to ~6,000 genes), with the remainder being non-assignable. The repetitive fraction included a number of as yet unknown sequences, and various TE families probably responsible for the differential expansion of the 3 constituent hexaploid wheat genomes were recognized. The sequencing and complete annotation of 136 chromosome 3B BACs, overall some 17 Mb of DNA assembled into 12 contigs spread over the entire chromosome, uncovered a total of 128 genes, 21 pseudogenes and 14 gene relicts, along with ~3,000 TEs. The TE sequence represented 83.5% of the whole, and showed that gene islands are small and scattered throughout the chromosome. This result has provided real data to support the notion that the whole genome needs to be sequenced to access the entire wheat gene space [Choulet et al., 2009]. The barley leaf rust resistance gene *Rph7* has orthologues on the wheat homoeologous group 3 chromosomes, and a comparison of the sequences of the equivalent regions of 3BS [Choulet et al., 2009], 3AS [Sehgal et al., 2009] and 3DS [Bartoš et al., unpubl. data] has provided a number of insights into the parallel evolution of regions related by descent from a common ancestor.

Positional Gene Cloning

Several projects have been initiated to isolate genes using the chromosome-specific BAC libraries. With respect to chromosome 3B, these include the *Fusarium* head blight gene *Fhb1* and the stem rust resistance gene *Sr2*. Neither of these genes is present in cv. Chinese Spring, from which the BAC library and the physical map were derived. Nevertheless, Liu et al. [2008] constructed a BAC contig spanning the *Fhb1* region using the library, while

McNeil et al. [2008] chose 12 BACs from the Chinese Spring 3B MTP, which represented 1.6 Mb of sequence surrounding *Sr2*, and used this to derive linked markers and to fine map the region. This was followed up by the construction of a customized library made from 10⁶ copies of chromosome 3B from cv. Hope (a carrier of *Sr2*); this library was used to form a localized contig spanning the target gene [Spielmeyer et al., 2009]. On chromosome arm 7DS, Šimková et al. [2010] have targeted the Russian wheat aphid resistance gene *Dn_{CI2401}* and the same library is being used to positionally clone a seed size QTL [Röder et al., 2009]. Meanwhile, on chromosome arm 7DL, the greenbug resistance gene *Gb3* has been selected as a cloning target [Perumal et al., 2010].

Genome Sequencing

The physical map of each individual chromosome will facilitate the sequencing of the bread wheat genome, chromosome arm by chromosome arm. The improved capacity of forthcoming sequencing technology [Mardis, 2008] will allow the parallel acquisition of the sequence of a large number of BAC clones, such as those forming an MTP. An alternative possibility would be to simultaneously sequence the pooled BAC making up a contig, since pilot experiments have shown that reads as short as 103 bp can be successfully assembled [Wicker et al., 2006]. As the expectation for individual read length continues to rise [Pettersson et al., 2009], improved throughput and thus more complete assembly of BAC clones can be anticipated. The possibility to sequence small, defined parts of the genome should simplify the assembly of large numbers of short reads and enable the reconstruction of the DNA sequence of whole chromosomes.

Conclusion

Our ability to produce BAC libraries from single chromosomes or chromosome arms allows the reduction of the large wheat genome into more manageable pieces, and thereby improves the prospects for developing wheat genomics. Chromosome-specific BAC libraries avoid the complication of polyploidy, and the smaller sequencing template which can be formed from them simplifies the assembly of a physical map. The work involved can be readily divided among the research community, so that each participant is given the responsibility to generate a sequence-ready physical map of a particular chromosome or chromosome arm. The libraries and resulting physical maps will provide the opportunity to better un-

derstand the structure of the wheat genome, and reveal the processes which have shaped the evolution of the polyploid genome. The elaboration of chromosome-specific BAC libraries from each wheat chromosome remains a major undertaking, necessitating the establishment of a dedicated platform to produce high quality libraries in a timely fashion. So far, 24 chromosome- and chromosome arm-specific BAC libraries, derived from 10 wheat chromosomes, have been constructed. This chromosome-based strategy will be equally suitable for other complex genomes, provided that the necessary technologies for chromosome flow sorting are either available or can be developed.

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