Construction and characterization of a bacterial artificial chromosome (BAC) library for the A genome of wheat

D. Lijavetzky, G. Muzzi, T. Wicker, B. Keller, R. Wing, and J. Dubcovsky

Abstract: A genomic bacterial artificial chromosome (BAC) library of the A genome of wheat has been constructed. *Triticum monococcum* accession DV92 was selected for this purpose because it is a cultivated diploid wheat and one of the parental lines used in the construction of a saturated genetic map. Leaves from this accession were used to isolate high-molecular-weight DNA from nuclei. This DNA was partially digested with restriction enzyme *Hin*d III, subjected to double size selection, electroeluted and cloned into the pINDIGO451 BAC vector. The library consists of 276 480 clones with an average insert size of 115 kb. Excluding the 1.33% of empty clones and 0.14% of clones with chloroplast DNA, the coverage of this library is 5.6 genome equivalents. With this genome coverage the probability of having any DNA sequence represented in this library is higher than 99.6%. Clones were sorted in 720 384-well plates and blotted onto 15 high-density filters. High-density filters were screened with several single or low-copy clones and five positive BAC clones were selected for further analysis. Since most of the *T. monococcum* BAC ends included repetitive sequences, a modification was introduced into the classical end-isolation procedure to select low copy sequences for chromosome walking.

Key words: bacterial artificial chromosome, BAC library, *Triticum monococcum*, wheat.

Résumé : Une banque génomique du génome A du blé a été produite dans des chromosomes bactériens artificiels (BAC). L'accession DV92 du *Triticum monococcum* a été choisie pour ces fins car il s'agit d'une lignée diploïde qui est cultivée et d'un des parents employés dans la construction de cartes génétiques saturées. De l'ADN de grand poids moléculaire a été extrait de noyaux des feuilles de ce génotype. Cet ADN a été partiellement digéré avec l'enzyme de restriction *Hin*dIII, assujetti à une double sélection en fonction de la taille, électroélué et cloné dans le vecteur BAC pINDIGO451. La banque comprend 276,480 clones dont les inserts ont une taille moyenne de 115 kb. En excluant 1,33 % de clones vides et 0,14 % de clones contenant de l'ADN chloroplastique, cette banque offre une couverture génomique équivalent à 5,6 génomes. Avec une telle couverture, la probabilité qu'une séquence y soit représentée excède 99,6 %. Les clones ont été disposés dans 720 boîtes à 384 puits et placés sur 15 membranes à haute densité. Ces membranes ont été criblées avec des clones à copie unique ou à faible nombre de copies et cinq clones BAC ont été choisis pour des analyses détaillées. Comme la plupart des extrémités de BAC du *T. monococcum* comprennent de l'ADN répété, une modification a été apportée à la méthode classique d'isolement des séquences terminales afin de sélectionner des séquences à faible nombre de copies pour les fins de marche chromosomique.

Mots clés : chromosome bactérien artificiel, banque de BAC, *Triticum monococcum*, blé.

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D. Lijavetzky,[1] G. Muzzi, and J. Dubcovsky.[2] Department of Agronomy & Range Science, University of California, Davis, CA 95616–8515, U.S.A.
T. Wicker and B. Keller. Institute of Plant Biology, Zollikerstr. 107, University of Zurich CH-8008, Switzerland.
R. Wing. Departments of Agronomy & Biological Sciences, Clemson University, SC 29634–0359, U.S.A.

[1]Present address: Instituto de Biotecnología, INTA, 1708 Morón, Buenos Aires, Argentina.
[2]Author to whom all correspondence should be addressed (e-mail: jdubcovsky@ucdavis.edu).

Introduction

Bacterial artificial chromosome (BAC) libraries (Shizuya et al. 1992) have been recently developed from many different crop plants (Frijters et al. 1997; Marek and Shoemaker 1997; Vinatzer et al. 1998; Woo et al. 1994; Zhang et al. 1996). These large insert libraries have been essential for physically mapping and for cloning valuable genes (Brommonschenkel and Tanksley 1997; Caetano-Anolles and Gresshoff 1996; Chen et al. 1997; Danesh et al. 1998; Wang et al. 1995).

However, there has been limited progress in the construction of BAC libraries for species with large genomes like wheat. The genome of hexaploid *Triticum aestivum* L. is 16 700 Mb (Bennett and Leitch 1995) and though the ge-

nome of diploid wheat *T. monococcum* L. is smaller (5600 Mb, Furata et al. 1986) it is still 13-fold larger than the genome of rice and 38-fold larger than the genome of *Arabidopsis thaliana* (L.) Heynh. This additional DNA is represented mainly by repetitive sequences that comprise more than 75% of the wheat genome (Flavell and O'Dell 1976). Because of the complexity of its genome, wheat genomics has lagged behind studies in other crops with smaller genomes.

It has been suggested that rice BAC libraries (Wang et al. 1995; Zhang et al. 1996) may be used for positional cloning of wheat or barley genes (Dunford et al. 1995; Kilian et al. 1995). However, this approach could be risky because of the high level of intra- and interchromosomal duplication in cDNA clones observed in the large genomes of the Triticeae (Dubcovsky et al. 1996). In addition, the substantial evolutionary divergence between rice and Triticeae species increases the possibility that the homoeologous targeted gene is not present in the selected rice BAC clone.

The construction and characterization of a *T. monococcum* BAC library is presented here including a detailed determination of insert size, chloroplast DNA contamination, and number of empty recombinant clones. This BAC library is being used in our laboratories for positional cloning of vernalization genes in diploid wheat and as a tool for map-based cloning in hexaploid wheat. Strategies to avoid the abundant repetitive sequences for chromosome walking and positional cloning are discussed.

Materials and methods

Preparation of the BAC vector

BAC vector pINDIGO451 was provided by Dr. H. Shizuya (California Institute of Technology). Supercoiled vector was prepared from 6 L of Luria-Bertani (LB) medium containing 30 μg/mL of chloramphenicol. Plasmid DNA was isolated according to Sambrook et al. (1989) followed by 2 cesium chloride gradient centrifugations. Ten micrograms of vector were completely digested with 100 U of *Hin*d III (New England Biolabs) and dephosphorylated with 10 U of HK phosphatase (Epicentre Technologies). In order to determine the efficiency of dephosphorylation, a self-ligation test was performed. Ligation with λ DNA digested with *Hin*d III was used to test the cloning efficiency of the prepared BAC vector. Aliquots of 100 ng of pINDIGO451 were stored at –80°C.

High-molecular-weight (HMW) DNA isolation

Plants of *Triticum monococcum* (DV92) were grown in a greenhouse for 6–8 weeks. Nuclei were isolated from leaves according to Zhang et al. (1995). Briefly, leaves were grounded in liquid nitrogen and nuclei were isolated, rinsed five times in washing buffer, resuspended in 2–3 mL in extraction buffer without β-mercaptoethanol, and embedded in 1.5% low-melting-point agarose plugs. Agarose plugs were incubated for 48 h in lysis buffer, and stored in TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 8.0) at 4°C. All buffers were prepared according to Zhang et al. (1995). HMW DNA integrity was tested by pulsed-field gel electrophoresis (PFGE) using a CHEF DRII apparatus (Bio-Rad) at 200 V, with a 1–40 s pulse, for 20 h at 12°C in 0.5× TBE buffer (0.09 M Tris–borate, 0.09 M boric acid, 0.002 M EDTA).

BAC library construction

Agarose plugs containing the HMW DNA were incubated overnight in TE (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0) at 4°C before the partial digestions. Before digestion with restriction enzyme, each agarose plug was chopped into small pieces with a sterile razor blade. Twenty-five microlitres of TEX (10:1 TE with 0.01% of Triton X–100) was added to each chopped plug to facilitate manipulation. Each chopped plug was incubated in 1× *Hin*d III restriction buffer (New England Biolabs) with 4 mM spermidine for 1 h in ice. One-half unit of *Hin*d III was added to the chopped plug and allowed to diffuse for 30 min in ice. For partial digestions, reactions were incubated for 15 min at 37°C and then stopped by adding one-tenth of the total volume of 0.5 M EDTA, pH 8.0. Partially digested DNAs were size-selected by PFGE. For the first size selection, DNAs were separated in 1% agarose gels at 200 V, with a 1 s to 40 s pulse ramp, for 20 h at 12°C in 0.5× TBE buffer. The region of the gel containing DNA between 125 kb and 350 kb was selected and cut using a lambda ladder as a molecular weight standard. For the second size selection, the agarose slice from the first size selection was loaded in a new 1% agarose gel. The conditions for the second size selection were 200 V, with 3 s pulse, for 20 h at 12°C in 0.5× TBE. The region of the gel containing the DNA was cut and the DNA was electroeluted from the agarose block following the method described by Strong et al. (1997) but using 600 μL of 1× TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA). After the electroelution, if DNA concentration was low, the DNA was concentrated by drop-dialysis against 0.5× TE with 20% polyethylene glycol (PEG) on a Millipore filter membrane for 2 h on ice.

Ligations were performed in 200 μL reactions containing 300 ng of partially digested DNA, 20 ng of linearized and dephosphorylated pINDIGO451 BAC vector, 1× TA buffer, 1 mM ATP and 6 units of T4 DNA ligase (Epicentre Technologies) at 16°C overnight. After ligation, the reactions were de-salted and concentrated by drop-dialysis against 0.5× TE with 20% PEG for 2 h on ice. Two microlitres of ligation reaction were used to eletroporate 20 μL of *Escherichia coli* ElectroMAX DH10B cells (BRL) using a BRL Cell-Porator system according the manufacturers' settings. After the transformation, the cells were resuspended in 500 μL of SOC medium (BRL, 2% Bacto tryptone, 0.5% Bacto Yeast extract, 10 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose, pH 7.0) and incubated for 1 h at 37°C with shaking at 225 rpm. The SOC medium with the cells was plated on LB plates containing 12.5 μg/mL chloramphenicol, 50 μg/mL X-Gal and 25 μg/mL IPTG (isopropyl–thiogalactoside), and incubated at 37°C overnight. White recombinant colonies were transferred to 384-well plates containing 75 μL of LB freezing buffer (Woo et al. 1994). The plates were incubated overnight at 37°C, duplicated and stored at –80°C.

BAC library screening

High-density filters were made using a Q-bot (Genetix, Dorset, U.K.). Each high-density filter contains 18 432 double-spotted clones to avoid false positives (Choi and Wing 1998). Hybridization and washes were performed as described earlier (Dubcovsky et al. 1994). For their first use, high-density filters were pre-hybridized overnight. Filters were autoradiographed for 2–72 h using Kodak BioMax MR1 film. Probes used for screening the library are listed in Table 1.

BAC DNA isolation

Individual BAC clones were inoculated in 3 mL overnight LB cultures with 12.5 μg/mL chloramphenicol. Supercoiled BAC DNA was isolated using an AutoGen 850α robot (AutoGen, Framingham, Mass.). BAC DNAs were digested with *Not*I to release the BAC insert. The digested clones were separated by PFGE

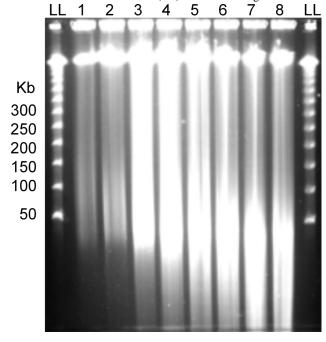**Table 1.** Summary of the *T. monococcum* BAC library hybridization screening.

Clone	N	Sources
BCD1427 (barley cDNA clone)	1	Anderson et al. (1989)
CDO708 (oat cDNA clone)	2	Anderson et al. (1989)
WG644 (wheat genomic clone)	5	Anderson et al. (1989)
pTdUCD1 (wheat low-molecular-weight glutenin)	4	Cassidy and Dvorak (1991)
pDY10A/K(–)(wheat high-molecular-weight glutenin)	1	Anderson et al. (1989)
TRI25–11 (triticin storage protein)	2	Rogowsky et al. (1991)
pTA31 (wheat puroindoline-a)	1	Gautier et al. (1994)
pGsp (wheat grain softness protein)	1	Rahman et al. (1994)
LRK10 (wheat receptor-like kinase)	4	Feuillet et al. (1997)
HvHAK1 (barley high affinity potassium transporter)	4	Santa-María et al. (1997)
HvHAK2 (barley high affinity potassium transporter)	1	Santa-María et al. (1997)
HvHAK3 (barley high affinity potassium transporter)	2	F. Rubio, ETSIA,[*] Spain
HvHAK4 (barley high affinity potassium transporter)	1	F. Rubio, ETSIA,[*] Spain
MWG79 (barley genomic clone)	2	Graner et al. (1991)
C1401 (rice cDNA clone)	4	Harushima et al. (1998)
R1618 (rice cDNA clone)	0	Harushima et al. (1998)
R2311 (rice cDNA clone)	2	Harushima et al. (1998)
R2628 (rice cDNA clone)	4	Harushima et al. (1998)
R2632 (rice cDNA clone)	3	Harushima et al. (1998)
S1912 (rice cDNA clone)	2	Harushima et al. (1998)
UCW1 (barley genomic clone)	1	D. Lijavetzky, UC Davis, U.S.A.
UCW4 (wheat genomic clone)	6	S. Marcucci, INTA,[†] Argentina
UCW12 (barley genomic clone)	8	M. Helguera, UC Davis, U.S.A.
pTacP3 (wheat chloroplast clone)	26	Ogihara et al. (1998)

Note: N, indicates the no. of positive BAC clones detected in four high-density filters.
[*]ETSIA, Escuela Técnica Superior de Ingenieros Agrónomos.
[†]INTA, Instituto Nacional de Tecnología Agropecuaria.

Fig. 1. PFGE (pulsed-field gel electrophoresis) analysis of partially digested *T. monococcum* HMW DNA. Lane 1, undigested DNA; lanes 2–8, DNA digested with 0, 0.5, 1, 1.5, 2, 4, and 6 units of *Hin*dIII respectively. PFGE conditions: voltage = 200 V, switch time = 1–40 s, run time = 20 h, T = 12ºC, 1% agarose gel in 0.5× TBE. The molecular weight standard is the Lambda ladder (LL) from New England Biolabs.

in a 1% agarose gel at 200 V, with a 5–15 s pulse ramp, for 14 h at 12ºC in 0.5× TBE buffer.
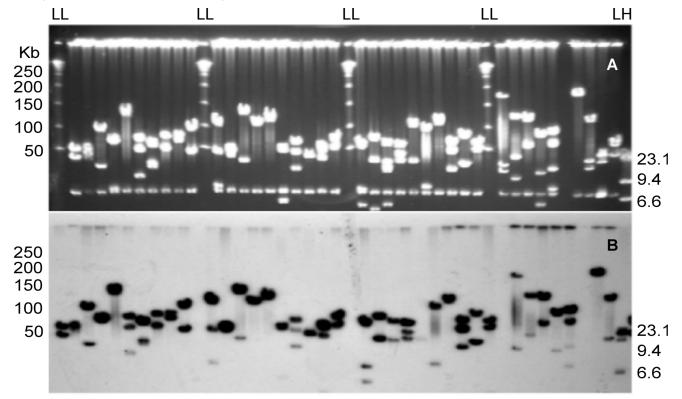
BAC end isolation and mapping

Plasmid rescue and inverse polymerase chain reaction (IPCR) were performed using the protocols described in Woo et al. (1994). Since all the isolated ends included highly repetitive sequences, an additional step was necessary to recover clones with low-copy-number sequences. Individual ends were digested with *Sau*3A I and cloned into pBluescript (Stratagene). Plasmid DNA isolated from the subcloned BAC ends was digested with *Eco*RI and *Xba*I, separated in a 1% agarose gel, and transferred to nylon membranes by Southern blot. Membranes were hybridized with [α-^{32}P]-labeled total genomic DNA of *T. monococcum* and positive clones were discarded. For the subclones obtained from the plasmid rescue, a second hybridization with total genomic DNA extracted from DH10B cells carrying the pINDIGO451 BAC vector was necessary to eliminate subclones containing vector DNA or bacterial genomic DNA fragments.

Results

Construction of a *T. monococcum* BAC library

After extraction of the HMW DNA, serial digestions were performed with 0–6 U of *Hin*dIII per 100 μL of chopped plug to optimize the conditions of the partial digestions. Increasing amounts of restriction enzyme showed a clear pattern of increasing digestion, with an optimum at 0.5 U of *Hin*dIII (Fig. 1). Almost no DNA degradation was observed

Fig. 2. Analysis of 39 randomly selected *T. monococcum* BAC clones digested with *Not*I. (A) Ethidium bromide stained PFGE gel (voltage = 200 V, switch time = 5–15 s, run time = 14 h, T = 12°C, 1% agarose gel in 0.5× TBE). (B) Southern blot of the gel in (A) after hybridization with radioactively labeled *T. monococcum* genomic DNA. Molecular weight standards are (LL) Lambda ladder (New England Biolabs) and (LH) *Hin*dIII-digested lambda DNA.

in the chopped plug used as a control (incubation at 37°C with restriction enzyme buffer and no restriction enzyme).

Three different size classes of partially digested HMW DNA from the first size selection gel were analyzed for transformation efficiency. Three agarose blocks with partially digested DNA were excised from the gel from the regions between 350 and 275 kb (region A), between 275 and 200 kb (region B), and between 200 and 125 kb (region C). The three agarose blocks were subjected to a second size selection with compressing pulse conditions, and then electroeluted. No recombinant colonies were obtained from DNA electroeluted from region A; 150 recombinants per µL of ligation product were obtained from region B; and 250–1000 recombinants per µL of ligation product were obtained from region C. Most of the *T. monococcum* BAC library consists of clones obtained from region C. Only 9690 clones are from region B.

When the compressed DNA bands from the second size selection were not used immediately, the agarose blocks were stored in TE (10 mM Tris–HCl, 10 mM EDTA, pH 8.0) at 4°C. Two techniques, electroelution (Strong et al. 1997) and digestion with Gelase (Epicentre technologies), were compared for their efficiency in recovering size-selected DNA from agarose blocks. Lower transformation efficiencies were obtained with DNAs isolated with Gelase than with DNAs isolated by electroelution in 1× TAE. The best results were obtained when the electroeluted DNA (average 0.5 ng/µL) was concentrated by drop-dialysis against PEG before the ligation reactions (average 3.5 ng/µL). After the ligation, the DNA was de-salted and concentrated by drop-dialysis. All transformations produced more than 95% recombinant white colonies.
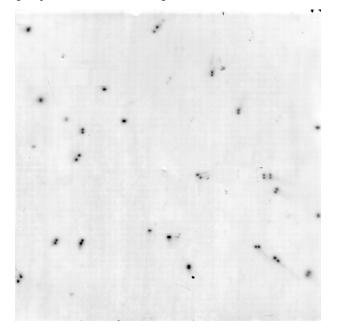
A total of 276 480 white colonies were transferred into 720 384-well plates and blotted onto 15 high-density filters.

BAC library characterization

To determine the average insert size of the library, DNAs from 913 random BAC clones were isolated. BAC DNAs were digested with *Not*I, fractionated by PFGE (Fig. 2A) and transferred by Southern blot to nylon membranes. The average insert size was estimated to be 115 kb with clones ranging from 33–233 kb and a standard deviation of 29 kb. The percentage of empty recombinant clones was 1.33%. Hybridization of the Southern blot with [α-^{32}P]-labeled total genomic DNA of *T. monococcum* showed that the inserts of all the analyzed BAC clones were wheat DNA (Fig. 2B). Eighty-five percent of the DNA fragments in Fig. 2B showed strong hybridization signals indicating presence of repetitive DNA sequences. This result was expected from the large proportion of repetitive elements present in the wheat genome.

The percentage of contamination with chloroplast DNA was determined by hybridizing 55 296 clones (three high-density filters) with [α-^{32}P]-labeled wheat chloroplast clone pTacP3 (Ogihara et al. 1998). Seventy-seven positive BAC clones were detected indicating a 0.14% of chloroplast contamination (Fig. 3).

Subtracting the empty BAC clones and the BAC clones with chloroplast DNA inserts, the *T. monococcum* library has a coverage of 5.6 genome equivalents, assuming a haploid genome size of 5600 Mb (Furata et al. 1986). With this genome coverage the probability of having any DNA sequence represented in the *T. monococcum* BAC library is higher than 99.6% (Clarke and Carbon 1976).

Four high-density filters (i.e., 73 728 clones) representing 1.6 genome equivalents of *T. monococcum* were screened by hybridization with 23 single- or low-copy-number probes (Table 1). An average of 2.6 positive clones per probe was obtained (0–8 positive clones). Only one probe (R1618 from rice) produced 0 positive clones (Table 1).

BAC end isolation and characterization

Nine ends from five BAC clones (five from IPCR and four from plasmid rescue) were isolated, transferred to nylon membranes by Southern blot and hybridized with [α-^{32}P]-labeled total genomic DNA of *T. monococcum*. All of them showed the presence of highly repetitive DNA sequences.

To obtain low-copy-number subclones, five of these ends were digested with restriction enzyme *Sau*3AI and subcloned into pBluescript (Stratagene). PCR amplified inserts were hybridized with [α-^{32}P]-labeled total genomic DNA of *T. monococcum,* and low-copy-number subclones were selected based on the relative strength of the hybridization signal compared with three control subclones of low-, medium-, and high-copy-number. A second hybridization step was used after the plasmid rescue to eliminate subclones containing pINDIGO451 BAC vector or bacterial genomic DNA fragments. The probe for this hybridization was obtained from a total genomic DNA extraction from DH10B cells carrying the pINDIGO451 BAC vector. Usually,

enough clones were analyzed to represent two- to three-fold the size of the original segment.

Discussion

Crucial steps in the construction of the library

To have a 99% or higher probability of recovering any sequence from a BAC library of *T. monococcum*, at least 224 250 BAC clones (average size 115 kb) are necessary. In order to obtain this large number of clones it was necessary to optimize every step in the construction of the library.

To optimize vector preparation, two sequential cesium chloride–ultracentrifugation steps were used instead of the column purification procedure used in most of the plant BAC libraries (Clarke and Carbon 1976; Danesh et al. 1998; Frijters et al. 1997; Marek and Shoemaker 1997; Mozo et al. 1998; Wang et al. 1995; Woo et al. 1994). A single batch of this supercoiled BAC vector was sufficient to clone more than 85% of the library. Transformations using this purified vector showed more than 95% white recombinant colonies and less than 1.4% empty clones. It was suggested that vector dephosphorylation leads to a rapid degradation of vector (i.e. 32 to 46 days) even during storage at –80°C (Danesh et al. 1998). However, transformation efficiency of this dephosphorylated vector was stable for nine months at –80°C.

Together with vector preparation, quality and quantity of partially digested HMW genomic DNA are the most critical factors in the construction of BAC libraries (Frijters et al. 1997; Wang et al. 1995; Zhang et al. 1995). Zhang et al. (1995) suggested that leaf tissues can be homogenized either by blending with a kitchen blender or by grinding in liquid nitrogen with a mortar and pestle. Both methods were compared here (data not shown) and the second one resulted in a lower proportion of degraded DNA in *T. monococcum*. This result parallels the observation of Zhang et al. (1995) of a larger proportion of intact nuclei in the liquid nitrogen homogenization (95%) than in the blender homogenization (57%). A modification introduced here to the protocol of Zhang et al. (1995) was the addition of three washing steps for the nuclei purification. These extra washes yielded a high quality and stable HMW DNA that was not degraded after nine months of storage at 4°C (Fig. 1, lane 1). This HMW DNA was stable even after chopping and incubating the HMW DNA plugs at 37°C for 15 min (Fig. 1, lane 2). The additional washing steps for the nuclei resulted also in a very low percentage of chloroplast DNA contamination (0.14%).

The quantity of input genomic DNA for the ligation reactions is as important as the quality of the HMW genomic DNA. A sufficient amount of DNA is needed to compensate for the lower transformation efficiency of the large inserts (Frijters et al. 1997; Leonardo and Sedivy 1990; Sheng et al. 1995). However, overloading the pulse field gel to increase the amount of partially digested DNA can result in an undesirable quantity of small size DNA fragments trapped within the large fragments (Frijters et al. 1997; Woo et al. 1994). To avoid this problem, a ramped pulse (1–40 s) was used in the first size selection followed by a standard second size selection. These PFGE conditions reduced the proportion of small DNA fragments trapped in the HMW DNA and resulted in an average insert size approximately equal to the size of the

excised agarose block from the first size selection. Different strategies were used to increase the yield of partially digested DNA for the ligations. First, agarose plugs were chopped in small pieces (i.e., microbead size) to increase the surface/volume ratio in order to improve the access of the restriction enzyme to the HMW DNA. Second, a concentration step was introduced after the electroelution of the partially digested DNA if DNA concentration was low.

Use of the *T. monococcum* BAC library for positional cloning

The suitability of a BAC library for positional cloning depends on the capability to recover clones from specific regions by screening with specific probes, and on the possibility of generating new probes from the ends of the selected BAC clones. The *T. monococcum* BAC library has adequate genome coverage, appropriate average insert size, a low percentage of chloroplast contamination, and few empty clones. However, the abundance of repetitive sequences creates additional problems for chromosome walking. This problem was overcome by the selection of single- and low-copy-number subclones from BAC ends as previously suggested by Woo et al. (1994). These small low-copy-number clones are being used to confirm BAC contigs assembled by fingerprinting, to screen the BAC library, and to map the BAC ends on the genetic maps.

In our laboratories the *T. monococcum* BAC library is being used for positional cloning of wheat vernalization genes (Dubcovsky et al. 1998) and leaf rust resistance genes (Feuillet et al. 1997), however, these will not be the only uses of this library. During recent years, molecular markers closely linked to numerous genes with agronomic importance have been identified in wheat and barley. These clones can be used to screen this library and to select BAC clones encompassing those genes or to generate additional markers in the targeted regions. This BAC library is publicly available and will be a long-term tool for genomic studies and for positional cloning in wheat (clones and filters can be requested at http://agronomy.ucdavis.edu/Dubcovsky/BAC-library/BAC_Home.htm).

Acknowledgements

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