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

**D. Lijavetzky[1], G. Muzzi[1], T. Wicker[2], B. Keller[2], R. Wing[3] and J. Dubcovsky[1 *]**

[1] Department of Agronomy & Range Science, University of California, Davis, CA 95616-8515, USA.

[2] Institute of Plant Biology, Zollikerstr. 107, University of Zurich, CH-8008, Switzerland.

[3] Departments of Agronomy & Biological Sciences, Clemson University, SC 29634-0359, USA.

[*] Corresponding author: Phone: (530) 752-5159, FAX: (530) 752-4361, E-mail: jdubcovsky@ucdavis.edu.

**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.

## Introduction

Bacterial artificial chromosomes (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 genome of diploid wheat *T. monococcum* L. is smaller (5,600 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 inter-chromosomal 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 liters of 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 µg of vector were completely digested with 100 units of *Hin*d III (New England Biolabs) and dephosphorylated with 10 units 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 DNA (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 (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 0.5x TBE buffer.

**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 µl 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 1x *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 1/10 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 0.5x 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 0.5x 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 x

TAE buffer. After the electroelution the DNA was concentrated by drop-dialysis against 0.5x 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, 1x TA buffer, 1 mM ATP and 6 units of T4 DNA ligase (Epicentre Technologies) at 16°C overnight. After ligation, the reactions were desalted and concentrated by drop-dialysis against 0.5x TE with 20 % PEG for 2 h on ice. Two µl of ligation reaction were used to eletroporate 20 µl of *E. 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) 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, and incubated at 37°C overnight. White recombinant colonies were picked 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). 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 h to 72 h using Kodak BioMax MR1 films. 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. BAC DNAs were digested with *Not* I to release the BAC insert. The digested clones were separated by PFGE in a 1% agarose gel at 200 V, with a 5 s to 15 s pulse ramp, for 14 h at 12ºC in 0.5 x 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*R I and *Xba* I, separated in a 1% agarose gel, and transferred to nylon membranes by Southern blot. Membranes were hybridized with [$\alpha$-$^{32}$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 extraction 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 to 6 units of *Hin*d III per 100µl 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 units of *Hin*d III (Fig. 1). Almost no DNA degradation was observed in the chopped plug used as a control (incubation at 37°C with 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) 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 1x 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 desalted and concentrated by “drop-dialysis”. All transformations produced more than 95% recombinant white colonies.

A total of 276,480 white colonies were picked 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 to 233 kb and a standard deviation of 29 kb. The percentage of empty recombinant clones was 1.33%. Hybridization of the Southern Blot with [$\alpha$-$^{32}$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 [$\alpha$-$^{32}$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 5,600 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/probe was obtained (0 to 8 positive clones). Only one probe (R1618 from rice) produced 0 positive clones (Table 1).

**BAC end isolation and characterization**

Nine ends from six BAC clones (five from IPCR and four from plasmid rescue) were isolated, transferred to nylon membranes by Southern blot and hybridized with [$\alpha$-$^{32}$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*3A I and subcloned into pBluescript (Stratagene). PCR amplified inserts were hybridized with [$\alpha$-$^{32}$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. The average size of the inserts of these clones was 552 bp (standard deviation 418 bp). 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, line 1). This HMW DNA was stable even after chopping and incubating the HMW DNA plugs at 37°C for 15 min (Fig. 1, line 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 s to 40 s) was used in the first size selection followed by a standard second size selection. This 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 here to increase the yield of partially digested DNA for the ligations. First, agarose plugs were chopped in small pieces (i.e. microbeads 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.

## The 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 posibility to generate 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.

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

| Clone | N° of BACs | 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/KS- (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, E.T.S.I.A., Spain |
| HvHAK4 (barley high affinity potassium transporter) | 1 | F. Rubio, E.T.S.I.A., 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, USA |
| UCW4 (wheat genomic clone) | 6 | S. Marcucci, INTA, Argentine |
| UCW12 (barley genomic clone) | 8 | M. Helguera, UC Davis, USA |
| pTacP3 (wheat chloroplast clone) | 26 | (Ogihara et al. 1998) |

**Figure captions**

**Fig. 1**: PFGE 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*d III respectively. PFGE conditions: voltage = 200 V, switch time = 1 s to 40 s, run time = 20 h, T = 12ºC, 1% agarose gel in 0.5x TBE. The molecular weight standard is the Lambda ladder (LL) from New England Biolabs.

**Fig. 2**: Analysis of 39 randomly selected *T. monococcum* BAC clones digested with *Not*I. (A) Ethidium bromide stained gel (voltage = 200 V, switch time = 5 s to 15 s, run time = 14 h, T = 12ºC, 1% agarose gel in 0.5x 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) lambda DNA *Hin*d III-digested.

**Fig. 3**: High-density filter screened with radioactively labeled chloroplast DNA clone pTacT3. Twenty-six positive clones were detected among the 18,432 double-spotted clones. Two of the eight double-spotted orientations are adjacent and may look as single-spotted in this reduced figure.