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ORIGINAL PAPER

B. Shen · D.M. Wang · C.L. McIntyre · C.J. Liu

A 'Chinese Spring' wheat (*Triticum aestivum* L.) bacterial artificial chromosome library and its use in the isolation of SSR markers for targeted genome regions

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Abstract A bacterial artificial chromosome (BAC) library was constructed from the bread wheat (*Triticum aestivum* L.) genotype 'Chinese Spring' ('CS'). The library consists of 395,136 clones with an estimated average insert size of 157 kb. This library provides an estimated 3.4-fold genome coverage for this hexaploid species. The genome coverage was confirmed by RFLP analysis of single-copy RFLP clones. The CS BAC library was used to develop simple sequence repeat (SSR) markers for targeted genome regions using five sequence-tagged-site (STS) markers designed from the chromosome arm of 3BS. The SSR markers for the targeted genome region were successfully obtained. However, similar numbers of new SSR markers were also generated for the other two homoeologous group 3 chromosomes. This data suggests that BAC clones belonging to all three chromosomes of homoeologous group 3 were isolated using the five STS primers. The potential impacts of these results on marker isolation in wheat and on library screening in general are discussed.

Introduction

Bacterial artificial chromosome (BAC) libraries are an invaluable resource for physical mapping (Mozo et al. 1999), gene cloning (Faris et al. 2003), comparative mapping and genome evolution studies (Dubcovsky et al. 2001; Sorrells et al. 2003), and also for developing polymorphic markers for targeted genome regions (Cregan et al. 1999). BAC libraries have been constructed for many plant species of agronomic importance. In wheat and its close relatives, BAC libraries have been reported for the diploid *Triticum tauschii* (D genome; Moullet et al. 1999), for the diploid *T. monococcum* (A^m genome; Lijavetzky et al. 1999), for the tetraploid *T. turgidum* (AB genome; Cenci et al. 2003), and for the hexaploid *T. aestivum* (ABD genome) using the genotypes 'Hartog' (Ma et al. 2000), 'Glenlea' (Nilmalgoda et al. 2003), and 'Chinese Spring' ('CS'; Allouis et al. 2003). Recently, chromosome specific BAC libraries based on 'CS' have also been reported (Janda et al. 2004; Šafář et al. 2004).

The spring wheat genotype 'CS' is widely used as the reference for genetic studies in wheat research. A vast range of aneuploids (Sears 1954; 1966), intervarietal single chromosome substitutions (Law et al. 1988), wheat–alien substitutions and additions (Gale and Miller 1988), and deletion stocks (Endo and Gill 1996) are based on this genotype. It is also the most commonly used genotype for the construction of Expressed Sequence Tags (ESTs) (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) and full-length cDNA libraries (Ogihara et al. 2004). Thus, 'CS' BAC libraries are another invaluable tool to add to the existing vast range of resources based on this genotype. Of the three gridded 'CS' libraries reported to date, the one reported by Allouis et al. (2003) has the largest insert size (130 kb) and provides 9.3 haploid genome equivalents. The library reported by Šafář et al. (2004) is chromosome-3B-specific. It has an average insert size of 103 kb and provides a six-fold coverage of this chromosome. The library reported by Janda et al. (2004) consists of BAC clones for three chromosomes, 1D, 4D, and 6D, with an average insert size of 85 kb and 3.4-fold coverage of the three chromosomes.

Of the numerous possible applications, BAC libraries have been used successfully in isolating molecular markers for targeted genome regions. This approach was demonstrated initially in soybean (Cregan et al. 1999) and has since been successfully used in several other

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B. Shen · D.M. Wang · C.L. McIntyre · C.J. Liu (✉)
CSIRO Plant Industry, 306 Carmody Road, St Lucia, Queensland, 4067, Australia
E-mail: chunji.liu@csiro.au

D.M. Wang
College of life science, Hebei Agricultural University, Baoding Hebei, China

species including sorghum (Bhattamakki et al. (2000), chickpea (Rajesh et al. 2004), and mungbean (Miyagi et al. 2004). In this paper, we report the construction of a new 'CS' BAC library and demonstrate the feasibility of its application in developing Simple Sequence Repeat (SSR) markers for targeted genome regions in this hexaploid species.

Materials and methods

'CS' BAC library construction

The BAC vector pBeloBAC II (developed by Drs. H. Shizuya and M. Simon at California Institute of Technology CA, USA) was used as the cloning vector, and the hexaploid wheat genotype 'CS' was used as the DNA donor. The procedure for library construction, including BAC vector preparation, isolation and partial digestion of high-molecular weight (HMW) genomic DNA, separation of partially digested DNA, size fractionation, ligation and transformation, were as described by Ma et al. (2000). The partial digestion of HMW DNA was carried out using the restriction enzyme *Hin*dIII (New England Biolabs, USA), and DNA fractions between 150 and 300 kb were used for cloning. The competent cells used for transformation were ElectroMAX DH10B (Invitrogen, Australia). Insert sizes of BAC clones from each ligation were determined by randomly testing between 18 and 30 clones per ligation. Plasmid DNA was isolated using the method described by Zhang et al. (1996). BAC inserts were released by *Not*I (Invitrogen) restriction and separated by Pulsed Field Gel Electrophoresis (PFGE) in 0.5×TBE buffer at 6 V/cm, 11°C, with 5–5 s pulses for 20 h. Recombinant BAC clones were picked using a robot (Genetix Qbot, UK) and stored in 384-well plates.

Filter preparation and screening

High-density BAC filters were prepared using a robotic system (Genetix Qbot). BAC clones were arrayed in a 3×3 pattern in duplicate onto 22.2 cm×22.2 cm Hybond N+ nylon membranes (Amersham, UK). The membranes were placed onto LB agar containing 12.5 μg/ml chloramphenicol and incubated at 37°C for 16–20 h until individual colonies grew to approximately 2 mm in diameter. The colonies were then lysed and DNA fixed onto the filter according to Zhang et al. (1996).

Three types of probes were used to screen the library. The first type consisted of 20 randomly selected single-copy Restriction Fragment Length Polymorphism (RFLP) clones kindly provided by Prof. M.D. Gale of the John Innes Centre, UK (Table 1). These probes were used to further estimate the genome coverage of the library. The second type consisted of two rice chloroplast clones (pRE10 and pRP11), kindly provided by Prof. M. Sugiura of the Nagoya University in Japan. These two rice chloroplast clones were used to estimate the percentage of chloroplast clones in the library. The third type consisted of PCR-amplified products of five sets of Sequence-Tagged Site (STS) primers (Table 2) developed for the 3BS chromosome arm (Liu and Anderson 2003). These STS probes were used to test the feasibility of using the library to develop SSR markers for targeted genome regions using the BAC technique in this hexaploid species. Methods for probe labelling and hybridization were as described in Humphry et al. (2002).

Developing SSR markers for the 3BS chromosome arm

To identify those BAC clones derived specifically from chromosome arm 3BS, inserts of all the positive BAC clones were used as templates for PCR-amplification using the original five sets of STS primers. Those BAC clones that gave PCR products were most likely derived from 3BS and were used to develop SSR markers.

Purified inserts from those candidate BAC clones were digested with the restriction enzyme *Sau*3A I (Biolabs), and ligated into *Bam*HI (BioLabs)-digested pBluescript II KS+ vector following the manufacturer's protocol. For each BAC clone used, 384 subclones were randomly picked and manually gridded onto Hybond-N+ membranes. The filters were incubated on LB agar containing 25 μg/ml ampicillin at 37°C overnight. The colonies were then lysed and DNA fixed onto filters according to the method described by Zhang et al. (1996).

For SSR development, the subclone-containing filters were screened with pooled oligonucleotide probes (including $(AC)_{15}$,$(AG)_{15}$, $(AAC)_{10}$, $(AAG)_{10}$, $(AGC)_{10}$, and $(AGG)_{10}$). Previous studies found that these six SSR motifs were among the most abundant in wheat (Wang et al. 1994; Gao et al. 2003). Hybridizations were carried out according to the method described by Cregan et al. (1994). Insert sizes of positive subclones identified were estimated by PCR amplification using M13 primers. Plasmid DNA from positive subclones was purified using a Ultraclean™ Mini Plasmid Prep Kit (Mo Bio Laboratories, CA, USA) and sequenced using M13 primers. Sequences flanking SSR motifs were used to design PCR primers using the primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The SSR primers were tested against the group 3 nullisomic–tetrasomic lines of 'CS' (Sears 1966) to determine their specificity to chromosome 3B. Those SSR markers located to chromosome 3B were further analysed against two hexaploid wheat genotypes, 'Janz' and 'Sumai 3', the parents of an F2 population available in our laboratory. Together with two existing 3BS SSR markers (gwm533 and gwm493) that are known to map to chromosome arm 3BS (Röder et al. 1998), new SSR markers that detected polymorphism between the two parental genotypes were further analysed against 96 F2 individuals from the cross. Linkage analysis was performed using the MapManager QTX program (Manly et al. 2001).

Table 1 The 20 PSR clones used to screen the 'CS' BAC library and positive BAC clones identified

RFLP probe	Positive BAC clones
psr92	221A20, 321E18, 500K7, 500M11
psr100	69D17, 88O10, 202J14, 313C8, 432M21
psr155	159L1,401E16, 401L12, 462N8
psr170	403C4, 411O24, 403M6, 433J2, 433K22, 453G15, 459F19
psr303	159L13, 386K6, 409C8, 434N15, 265K18
psr313	37O8, 49H10, 159L11, 184G24, 303M2, 389N15, 391G22, 451O8
psr335	8C3, 47H12, 55L22, 187K13
psr389	17D19, 214G16, 461A19, 460L6
psr546	37H2, 105E19, 159L3, 184H2, 453M23, 504J11
psr584	62D20, 192I16, 192M18, 266J6
psr605	7D8, 16M20, 16I23, 108L18, 147P19, 477H5
psr630	163P14, 184C16, 202H4, 383K19
psr649	69F17, 263C12, 264L21
psr662	267K17, 374C10, 409P3, 462L14, 467A23, 469N12
psr687	383J11, 400L15, 428N17, 437M4
psr694	37O18, 47G12, 159L10, 184G24, 178K13, 266J6
psr907	275K13, 299O22,449L12,
psr912	81I13, 128M7, 221N5, 212L5, 302B9
psr921	221A21, 296P10, 331E18, 400P3, 476I20, 494J22, 494G10
psr941	302B19, 367J17, 374N10, 446D16, 460C8, 464F15, 494G22

Results

'CS' BAC library

'CS' BAC clones were obtained from a total of ten ligations using a single preparation of HMW DNA. The only difference between the ligations was the molar ratios of DNA and vector used which varied from between 1:5 to1:10. In total, 395,136 clones were picked and arrayed in 1,029 384-well plates.

To estimate the sizes of the inserts contained in these clones, 247 random clones from the ten different ligations were analysed. About 92% of these clones contained inserts, ranging from 40 to 350 kb with an average insert size of 157 kb. The distribution of the insert sizes is shown in Fig. 1.

The percentage of chloroplast clones in the library was estimated by screening two rice chloroplast-specific probes (pRE10 and pRP11). The two probes detected 87 positive BAC clones. Thus, the percentage of chloroplast clones contained in the wheat BAC library is estimated to be approximately 0.5%.

To check the accuracy of the genome coverage of the BAC library, 20 randomly selected single-copy wheat RFLP probes were used to screen 21 high-density filters containing 193,536 BAC clones. These probes detected between 3 and 7 BAC clones each, giving a total of 102 positive clones (Table 1), with an average of 5.1 clones per probe.

Table 2 STS primers used, positive BACs identified and new SSRs developed and their chromosomal locations

STS used[a]	BACs (kbp)	New SSR	Motif	Forward primer (5′–3′)	Reverse primer (5′–3′)	Location[a]
STS3B-17/49	88M6(180)	CS-SSR1	(TC)29	TCCTGCCCCACCTTATTTTC	AACGAGAAAGACGAGGGGCT	3A
		CS-SSR3	(TC)28	CCATCGTACGTCTCTGCTCA	CGGGATCTGAACGAGAAAGA	3A
	411B2(175)	CS-SSR5	(GA)19	GATCGTGCCAAATCAAGAACG	CGCCTCACTTGTTTCTTGAC	?
		CS-SSR21	(GA)19	TGCCAAATCAAGAACGAGAG	TGGAATTCTTTTACTTCTTC AGTTTG	?
STS3B-47	283E24(160)	CS-SSR2	(TC)30	CCGAAAGGTCGAACATGAGT	GGTACGTTACTTGCCCGAGA	?
		CS-SSR4	(TC)18	CTATCCATGACGAGGAGGGA	GTGCCATAGAGGGAACACGA	?
		CS-SSR7	(CA)31	GATGTGTCGCTCCCATTTAGT	GCCAAACTGCCTCACTCCAA	3A, 3B
		CS-SSR20	(AG)29	TTTGGGTGGAGGTGTTGAGT	TGGCGATCTGATGCATCTCT	3B
	476I20(165)	CS-SSR15	(C)21	GTGGGAAGGAGTAAGTCAGG	CCGTCGTTGTCTTCCTTCTC	3D
		CS-SSR17	(C)13	ATTGCTGGCTTCACTCTGCT	GCTATGATGCGGCTCTTAAGAT	?
STS3B-49	15A3(150)	CS-SSR6	(TTG)10	CCATCGTACGTCTCTGCTCA	CGGGATCTGAACGAGAAAGA	3A
	88M6(180)	CS-SSR11	(TCC)6	CTTGTGGGTTATCACGCTGC	ACCGCCAGTAGATCGTCATC	?
		CS-SSR19	(GA)28	AACGAGAAAGACGAGGGGCT	AGCATGCTTGCCCATGTCT	?
STS3B50/66	352D18(180)	CS-SSR8	(GTT)8	TCTTAGCGGTGTGTGTGGAG	TCCTACTGCACGGTTCAA AAG	3D
		CS-SSR14	(CA)31	GCCGATTTATAGGCATAAT GTCA	CCATTCCAA AAGTATTTCAAT GTGT	3D
	357P12(180)	CS-SSR9	(CAA)8	AACAGGTGAAGATGGGTTGCA	AGGAGAGGGCTGTGGAAAATA	?

[a] '?' represents those SSR products that were unable to be located to any of the three homoeologous group 3 chromosomes

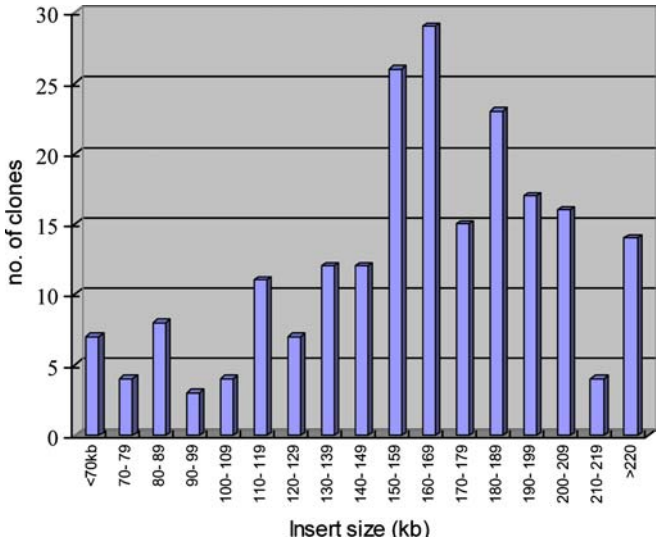

Fig. 1 The distribution of insert sizes of 247 random 'CS' BAC clones

Isolating BAC clones derived from chromosome arm 3BS

Screening the 21 high-density filters containing 193,536 BAC clones using the five STS probes identified 27 positive BAC clones. The STS primers successfully amplified products in seven of the 27 BAC clones, indicating that these BAC clones are likely to originate from chromosome arm 3BS. The insert sizes of the seven BAC clones varied from 150 to 180 kb, and they contain 1,190 kb wheat DNA in total (Table 2).

All seven BAC clones were subcloned, and 384 random subclones from each BAC were isolated. These subclones contained inserts ranging from approximately 75 to 3,000 bp. The subclones were screened with six selected SSR motifs, producing 38 positives. All of the putative SSR-containing subclones were sequenced, and 18 of them were found to contain SSR sequences. For two of the subclones, the SSR sequences were located close to the cloning sites, leaving one side of the flanking sequence too short for primer design. Primers for the remaining 16 SSR sequences were designed (Table 2) and tested against the group 3 nullisomic–tetrasomic lines of 'CS'. Two sets of these primers amplified products from chromosome 3B, three amplified 3A products, and three amplified 3D products; chromosomal locations of the remaining eight SSRs could not be determined (Fig. 2; Table 2).

One of the two 3B SSRs, CS-SSR7, detected a polymorphism between 'Janz' and 'Sumai3'. This SSR and two known 3BS SSRs (gwm533 and gwm493) were screened over all of the F2 progeny of this population and the polymorphisms scored. CS-SSR7 was found to be located 9.3 cM from gwm493, and 23.5 cM from gwm533.

Discussion

Construction of the CS BAC library

The CS BAC library constructed in this study contains 395,136 clones and has an average insert size of 157 kb. Based on the estimated genome size of hexaploid wheat of 16,700 Mb (Bennett and Leitch 1995), this BAC library provides an estimated 3.4-fold genome equivalents of the hexaploid wheat genome when the percentage of chloroplast sequences and empty clones are taken into consideration. This is supported by results from screening the 20 single-copy RFLP clones. These RFLP clones detected an average of 5.1 positives BAC clones each from the 193,536 BAC clones screened. This translates into 10.4 positive BAC clones for each of the probes when the whole library is used and 3.5 BAC clones for each of the three wheat genomes.

It is of note that the chloroplast content of the library could be underestimated using the rice chloroplast-specific probes. Although these rice probes have been used to isolate chloroplast sequences from legume species at the same stringency level applied during the present hybridization experiments (Liu and Musial 2001), the sequence differences between them and the wheat chloroplast genome mean that some chloroplast-containing clones might not be detected. By contrast, the genome coverage derived from the 20 RFLP clones could be overestimated due to the possibility that some of the clones may not be single copy.

The percentage of empty clones contained in this 'CS' library is approximately 8% which is relatively high compared to other BAC libraries reported in this species (Allouis et al. 2003; Nilmalgoda et al. 2003). One of the possible contributing factors to this could be the method of colony picking. We used blue/white colour selection for differentiating recombinant and non-recombinant clones. As seen in many other types of libraries where this colour system was used, the colour intensity of the 'CS' BAC clones varied, ranging from strong blue to pure white. To avoid missing too many white (recombinant) clones, the robot settings may have allowed the picking of some colonies with a light blue colour. The blue colour for some of the colonies could become much stronger with a prolonged incubation. This, however, was not always possible. Prolonged incubation would produce larger colonies, leading to larger proportions of joined colonies that make robotic picking difficult.

One of the difficulties in constructing and exploiting a hexaploid wheat BAC library is the large genome size of this species. Even though the average insert size of the library we obtained in this study is the largest among wheat libraries reported so far, the library represents only 3.4 genome equivalents. Although proven to be useful in the development of molecular markers, these clones alone are not adequate for projects such as

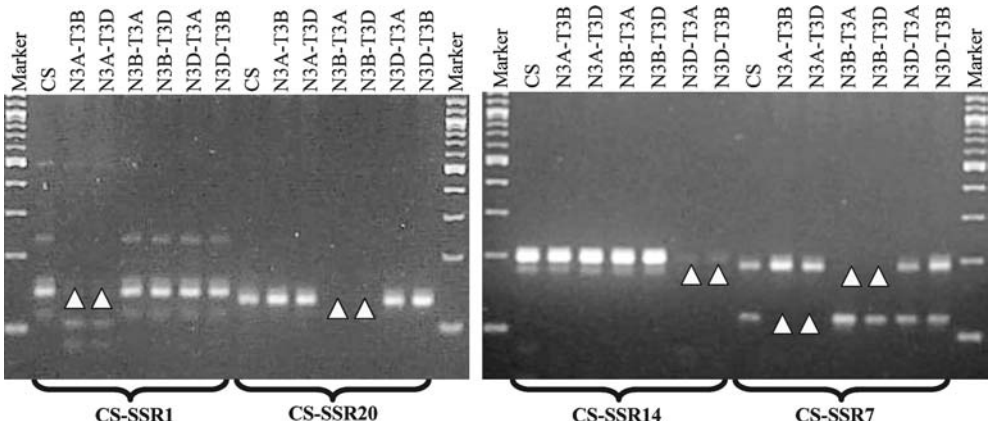

Fig. 2 Banding patterns of four new SSR markers analysed using 'CS' group 3 nullisomic-tetrasomic lines. The absence of 'CS' fragments are marked by *triangles* and indicate the chromosome specificity of the SSR in question. Marker used was 100 bp DNA ladder

physical mapping or map-based gene cloning. For the latter applications, combining all of the available libraries based on this genotype, including the library reported by Allouis et al. (2003), the subgenomic library reported by Janda et al. (2004), and the chromosome 3B library reported by Šafář et al. (2004), is necessary.

Developing SSR markers for targeted genome regions in hexaploid wheat

Previous studies have shown that even 'single-copy' RFLP probes usually hybridize to DNA fragments of all three chromosomes belonging to a homoeologous group in hexaploid wheat (Chao et al. 1989; Liu et al. 1992). The hexaploid nature of wheat thus makes individual chromosome identification more complicated. One of the possible methods to identify individual chromosomes is using PCR-amplification, as 'locus-specific' PCR primers are common in wheat (Devos et al. 1995; Röder et al. 1998).

Of the 27 positive BAC clones detected using STS probes in this study, seven produced PCR products using the five sets of STS primers. The seven BAC clones contain 1,190 kb wheat DNA from which 18 SSR sequences were isolated with the use of six SSR motifs. On average, one SSR was identified every 66 kb of BAC DNA. This frequency is much lower than an SSR every 17.2 kb DNA previously found in this species (Gao et al. 2003). The discrepancy in SSR frequency in the two studies is likely to be due to the smaller number of SSR motifs tested in this study. It may also be due to the different ESTs analysed and the specific BAC clones assessed.

As all of the five sets of STS primers amplified 3BS products (Liu and Anderson 2003), it would not be unreasonable to assume that most, if not all, of the seven BAC clones originated from this chromosome arm. However, of the 16 SSRs developed from the seven BAC clones, only two were mapped to chromosome 3B. Similar numbers of new SSR markers were located to each of the other two members of this homoeologous group, chromosomes 3A or 3D (Table 2). These data seem to suggest that the five sets of STS primers used in this study, although all mapped to chromosome arm 3BS (Liu and Anderson 2003), could amplify BAC clones from all of the three homoeologous chromosomes with similar effectiveness. This can have serious consequences. First, it would result in a greatly reduced efficiency in isolating markers for targeted genome regions in hexaploid wheat. Second, it suggests that library screenings in general based on PCR amplification could produce a large amount of false positives in polyploid species. Further investigation of this using a diverse set of 'locus-specific' primers would seem to be warranted.

It is of interest to note that although derived from different ESTs, STS3B-50 and STS3B-66 detected the same two BAC clones (Table 2), indicating each of the two BAC clones to contain both of the EST sequences. Similarly, two common BAC clones were amplified by STS17 and STS49, again indicating the two ESTs from which these two STS primers were derived are likely to physically map closely to each other. This data suggests that the major locus conditioning Fusarium head blight (FHB) resistance may reside in a gene-rich region. These STS markers map closely to the peak of the major FHB locus (Liu and Anderson 2003), which make these BAC clones containing the two EST sequences interesting targets for isolation of the FHB-resistant gene(s).

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