Theor Appl Genet (2006) 113: 23–32
DOI 10.1007/s00122-006-0265-7

ORIGINAL PAPER

Jiuhuan Feng · Brady A. Vick · Mi-Kyung Lee
Hong-Bin Zhang · C. C. Jan

Construction of BAC and BIBAC libraries from sunflower and identification of linkage group-specific clones by overgo hybridization

Received: 13 December 2005 / Accepted: 9 March 2006 / Published online: 13 April 2006

Abstract Complementary BAC and BIBAC libraries were constructed from nuclear DNA of sunflower cultivar HA 89. The BAC library, constructed with *Bam*HI in the pECBAC1 vector, contains 107,136 clones and has an average insert size of 140 kb. The BIBAC library was constructed with *Hin*dIII in the plant-transformation-competent binary vector pCLD04541 and contains 84,864 clones, with an average insert size of 137 kb. The two libraries combined contain 192,000 clones and are equivalent to approximately 8.9 haploid genomes of sunflower (3,000 Mb/1C), and provide a greater than 99% probability of obtaining a clone of interest. The frequencies of BAC and BIBAC clones carrying chloroplast or mitochondrial DNA sequences were estimated to be 2.35 and 0.04%, respectively, and insert-empty clones were less than 0.5%. To facilitate chromosome engineering and anchor the sunflower genetic map to its chromosomes, one to three single- or low-copy RFLP markers from each linkage group of sunflower were used to design pairs of overlapping oligonucleotides (overgos). Thirty-six overgos were designed and pooled as probes to screen a subset (5.1×) of the BAC and BIBAC libraries. Of the 36 overgos, 33 (92%) gave at least one positive clone and 3 (8%) failed to hit any clone. As a result, 195 BAC and BIBAC clones representing 19 linkage groups were identified, including 76 BAC clones and 119 BIBAC clones, further verifying the genome coverage and utility of the libraries. These BAC and BIBAC libraries and linkage group-specific clones provide resources essential for comprehensive research of the sunflower genome.

Communicated by Q. Zhang

J. Feng
Department of Plant Sciences, North Dakota State University, Fargo, ND 58105, USA

B. A. Vick · C. C. Jan (✉)
US Department of Agriculture, Agricultural Research Service, Northern Crop Science Laboratory, PO Box 5677, Fargo, ND 58105, USA
E-mail: janc@fargo.ars.usda.gov
Tel.: +1-701-2391319
Fax: +1-701-2391346

M.-K. Lee · H.-B. Zhang
Department of Soil and Crop Sciences and Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, TX 77843-2123, USA

Introduction

Sunflower (*Helianthus annuus* L.) is one of the world's leading edible oilseed crops, after oil palm, soybean, and rapeseed. Sunflower oil is considered a premium cooking oil for human consumption because of its high concentration of unsaturated fatty acids, oleic and linoleic, and its low concentration of linolenic acid (Dorrell and Vick 1997; Leon et al. 2003). Molecular breeding and genetic research provide opportunities for enhanced genetic improvement of many crops, including sunflower. To facilitate this research, a large number of molecular markers have been developed and several genetic linkage maps have been produced for sunflower, including restriction fragment length polymorphism (RFLP) (Berry et al. 1994, 1995; Gentzbittel et al. 1995, 1999; Jan et al. 1998), amplified fragment length polymorphism (AFLP) (Peerbolte and Peleman 1996; Gedil et al. 2001; Langar et al. 2003), and simple sequence repeat (SSR) (Tang et al. 2002; Yu et al. 2003). Using these DNA markers, numerous genes have been mapped (Gentzbittel et al. 1998; Horn et al. 2003; Pérez-Vich et al. 2005).

Genetic mapping of genes makes it possible to isolate genes of agronomic importance by map-based cloning. However, it requires a fine genetic map and a large-insert DNA library. Therefore, the large-insert DNA libraries have been developed and utilized in recent years, including yeast artificial chromosome (YAC), P1-derived artificial chromosome (PAC), plasmid-based clone (PBC), plant transformation-competent artificial chromosome (TAC), bacterial artificial chromosome (BAC)

and binary bacterial artificial chromosome (BIBAC) (Wu et al. 2004a) libraries. Although all types of large-insert DNA libraries have provided tools for positional cloning and advanced genomics research (Burke et al. 1987; Shizuya et al. 1992; Ioannou et al. 1994; Hamilton 1996, 1999; Tao and Zhang 1998; Liu et al. 1999; Chang et al. 2001), bacteria-based large-insert libraries such as BAC and BIBAC libraries have emerged as the preferred cloning systems for genomics research due to their low frequencies of chimeric clones, stable maintenance of large-insert (100–300 kb) DNA clones, and ease of cloned DNA purification and manipulation (Shizuya et al. 1992; Tao and Zhang 1998; Chang et al. 2003; Song et al. 2003; Wu et al. 2004a; Ren et al. 2005).

Large-insert BAC and BIBAC libraries have been constructed for more than 30 plant species. These species not only encompass the model plants and staple crops, such as *Arabidopsis thaliana*, rice, maize, and wheat (Wang et al. 1995; Zhang et al. 1996a; O'Sullivan et al. 2001; Tao et al. 2002; Chang et al. 2003; Nilmalgoda et al. 2003), but also extend to many other species, such as peach, papaya, melon, and garlic (Luo et al. 2001; Ming et al. 2001; Georgi et al. 2002; Lee et al. 2003). The first BAC library reported in sunflower (Gentzbittel et al. 2002), constructed with *Hin*dIII in the pBeloBAC11 vector, had a fourfold to fivefold genome coverage with an average insert size of 80 kb. Another sunflower BAC library was constructed using the same enzyme and vector system, with a 1.9-fold genome coverage and an average insert size of 60 kb (Özdemir et al. 2004). These libraries have proven useful for several aspects of sunflower genomic research; however, their smaller insert sizes and low genome coverage limit their utility for chromosome walking and construction of physical maps (Cai et al. 1995; Wu et al. 2004a, b; Ren et al. 2005). Moreover, libraries constructed by using a single enzyme are more likely to have a bias in genome coverage because of unevenness in the distribution of restriction enzyme cleavage sites along chromosomes (Tao et al. 2002; Chen et al. 2004; Wu et al. 2004b). Hence, construction of new BAC libraries that have significantly larger insert sizes and deeper genome coverage by using different restriction enzymes is essential for comprehensive genomics research of sunflower.

Sunflower has a genome of about 3,000 Mb/1C (Arumuganathan and Earle 1991); therefore, a huge number of clones are needed to construct a deep genome coverage library. For this reason, an efficient screening technique to address target clones is of the foremost importance for the utility of its libraries in genomics research. Traditionally, BAC libraries are screened by hybridizing high-density clone filters using radioactively labeled probes of target DNA, such as PCR products or cloned DNA fragments. However, these probes often contain several hundred base pairs of DNA and are more likely to carry repeat sequences that cause cross hybridization. This is especially true for the species with large genomes such as sunflower. Therefore, an alternative hybridization approach using overlapping oligonucleotides (overgo) as probes has been developed and used for library screening in animals (Cai et al. 1998; Royce-Tolland 2001; Ren et al. 2003; Romanov et al. 2003) and humans (Han et al. 2000). This technique has also been used in a few plant species such as rice (Chen et al. 2002), maize (Gardiner et al. 2004), and peanut (Yüksel and Paterson 2005).

Overlapping oligonucleotides, termed ''overgos'', are primer pairs that span about 40 bp in length and are usually constituted from two 24-bp oligonucleotides that have an 8-bp overlapping region at the 3′ ends. This feature allows the overgo primer pair to prime on each other and synthesize their complementary strands with radioactive nucleotides by the Klenow filling method. The major advantage of overgo probes over conventional probes for library hybridization is that the sequences for designing overgos can be selected, and thus repeated sequences present in a conventional DNA fragment probe can be avoided; therefore, the cross-hybridization problem that is frequently associated with large-genome DNA library screening can be minimized. Because of this advantage, overgo hybridization combined with the probe pooling strategy (Cai et al. 1998; Chang et al. 2001; Tao et al. 2001; Romanov et al. 2003) has emerged as the method of choice in high-throughput BAC library screening for clone identification and physical gene mapping.

Large-insert BAC clones have been widely used as probes for fluorescence in situ hybridization (FISH). Since FISH allows direct observation of a probe hybridization signal on a chromosome, it has become a useful technique for chromosome identification and genome physical mapping (Jiang and Gill 1994; Dong et al. 1998). It has been shown that a probe containing less than 10 kb of single-copy DNA generates a hybridization signal that is difficult to detect on metaphase chromosomes in plants (Jiang et al. 1995). However, a large-insert DNA probe derived from a BAC clone (about 100–300 kb) can create a strong FISH signal on metaphase chromosomes because of their large hybridization targets. Therefore, construction of a BAC library with an aim to conduct FISH using BAC clones as probes was an important objective for cytogenetic studies of potato (Dong et al. 2000; Song et al. 2000), barley (Lapitan et al. 1997), and garlic (Lee et al. 2003). Similarly, we are utilizing the linkage group-specific BAC or BIBAC clones identified in this study to identify individual chromosome of sunflower (Feng et al. 2005).

In this paper, we report the construction of one BAC library and one BIBAC library from the widely used inbred line HA 89 using two restriction enzymes (*Bam*HI, *Hin*dIII) and two vectors (pECBAC1, pCLD04541). These libraries have much larger insert sizes and deeper coverage than the existing sunflower BAC libraries. Using the large-insert libraries, we identified a set of sunflower linkage group-specific BAC or BIBAC clones by overgo hybridization, and are utilizing this information to characterize a set of sunflower trisomic lines. These new BAC and BIBAC libraries, along with

existing BAC libraries, will provide resources essential for more comprehensive sunflower genomics research.

Materials and methods

Plant materials

Inbred line, HA 89, was used for BAC and BIBAC library construction. HA 89 was released by the USDA-ARS and the Texas Agricultural Experiment Station in 1971 as a maintainer line. It has been used extensively as a parent in applied hybrid breeding program and to develop mapping populations (Gentzbittel et al. 1995; Jan et al. 2002; Langar et al. 2003; Burke et al. 2004). Therefore, the libraries constructed from HA 89 could be used directly to isolate genes and QTLs identified in the populations. Plants were grown in a growth chamber under typical condition, and then kept in the dark for one week prior to sample collection to minimize the starch and polysaccharide concentration which may affect quality megabase-sized nuclear DNA isolation. Leaves were collected from 2-week-old seedlings, weighed, frozen in liquid N_2, and stored at −80°C.

Preparation of the vectors

Two types of vectors, F plasmid-based BAC vector pECBAC1 and P1 plasmid-based BIBAC vector pCLD04541 (Wu et al. 2004a; Ren et al. 2005), were used to construct the libraries. The vector DNAs were isolated from the 1- to 2-L overnight cultures of pECBAC1 and pCLD04541 vector cells by the alkaline lysis method, purified by cesium chloride (CsCl) gradient centrifugation, completely digested with *Bam*HI and *Hin*dIII, respectively, and dephosphorylated with calf intestinal alkaline phosphatase (CIAP) (Invitrogen, USA) (Zhang 2000; Wu et al. 2004a).

Construction of BAC and BIBAC libraries

The libraries were constructed according to the protocol developed by Zhang (2000) (also see Wu et al. 2004a; Ren et al. 2005) with minor modifications. Briefly, nuclei were prepared from 80 g of frozen leaves. The leaves were ground into fine powder in liquid nitrogen, then transferred into 1× HB buffer, and washed at least four times using the buffer. The resulting nuclei were embedded in 1% low-melting-point (LMP) agarose plugs, which provided a solid matrix to prevent the DNA from physical shearing after nuclei lysis. The nuclei plugs that produced high-quality megabase DNA were colorless and slightly clear. Plugs that were milky-white (due to too much starch) or dark-brown (due to too much polyphenolics) did not result in high-quality megabase DNA. The plugs were incubated in lysis buffer (0.5 M EDTA; pH 9.0–9.3, 1% sodium lauryl sarcosine) containing proteinase K (0.3 mg/ml proteinase K) overnight at 50°C, washed three times in ice-cold TE (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0) plus 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to purify the megabase DNA, respectively, and then stored in TE at 4°C.

Each plug was cut into nine slices of equal size with a glass slide cover, followed by partial digestion with either *Bam*HI or *Hin*dIII and analysis by pulsed-field gel electrophoresis (PFGE) using a CHEF DRIII system (Bio-Rad, Richmond, CA) to determine the optimal enzyme concentration. Large-scale partial digestion was then carried out with 8 to 10 plugs using the selected optimal enzyme concentration. The plug slices containing partially digested DNA were size-selected on a 1% pulse-field agarose gel in 0.5× TBE under the following condition: 90-s switch time, 6 V/cm, 12.5°C, for 14 h, and then 4 V/cm, with a 5-s switch time for 5 h. In our procedure, only one size-selection was performed, and the DNA fractions ranging from 150 to 250 kb were excised from the gel, recovered by electroelution, and dialyzed in 0.5× TE. The concentration of the eluted DNA was estimated on a 1% agarose gel using known concentrations of λ DNA as a standard. The size-selected DNA fragments were ligated into the cloning vector. For the pECBAC1 vector, a molar ratio of 3:1 (vector:insert) was used, and for the pCLD04541 vector, a molar ratio of 4:1 (vector:insert) was used. The ligation reaction was performed in a mixture containing 100 µl of insert DNA and appropriate vector DNA with 2 units of T4 DNA ligase (Invitrogen, USA) at 16°C for 10 h.

The ligation mixture was used directly for transformation or aliquoted and stored at −20°C for later transformation. Approximately 1.0–1.5 µl of the ligation mixture was used to transform 20 µl of the *E. coli* strain ElectroMax DH10B cells by electroporation using a cell-porator *E. coli* pulser 11613 (Gibco BRL, USA) at the medium setting (~2.5 kV). Transformed cells were incubated in 1 ml SOC medium (Sambrook et al. 1989) for 1 h at 37°C, plated on LB agar medium with X-gal, IPTG, and chloramphenicol for the pECBAC1 vector or tetracycline for pCLD04541 vector (Zhang 2000; Wu et al. 2004a; Ren et al. 2005). The cultures were incubated at 37°C for about 24 h to allow the colonies to grow until the colony turned either blue or white. Blue colonies indicate no insert, while the white colonies were potential recombinants and thus selected as BAC or BIBAC clones.

To estimate the insert sizes of the clones, DNA from randomly selected BAC and BIBAC clones was isolated by the alkaline lysis method, completely digested with *Not*I, and separated on CHEF (contour-clamped homogeneous electric field) gels (Zhang 2000; Wu et al. 2004a; Ren et al. 2005). The ligation that produced clones with an average insert size of 130 kb or larger was selected for large-scale transformation and library assembly. The BAC or BIBAC clones were manually arrayed into 384-well microtiter plates, with each well containing 65 µl of cell freezer storage medium with appropriate antibiotics

(Zhang et al. 1996a; Zhang 2000). The plates were incubated at 37°C overnight to allow the clones to grow, then duplicated into three copies, and stored in freezers at −80°C for long-term use.

Preparation of high-density clone filters for library screening

The BAC and BIBAC clones of the libraries were robotically gridded on 22.5×22.5-cm Hybond N+ membranes (Amersham, USA) in a 4×4 format using the GeneTAC™ G^3 Robotic Workstation (Genomic Solutions, Inc., USA). A total of 6×8×384 = 18,432 clones were double-spotted on each high-density clone filter. The filters were processed according to a standard alkaline lysis method (Zhang et al. 1996a; Zhang 2000), baked at 80°C for 2 h and stored at 4°C before use.

Probe labeling

One to three single- or low-copy RFLP markers were selected from each linkage group of the sunflower (Jan et al. 1998). Overgos were designed for each RFLP marker according to their sequences using the Overgo 1.02i program (http://www.mouse-genome.bcm.tmc.edu) and processed by following Romanov et al. (2003). The overgos were synthesized and then labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP, following the method developed by J. D. McPherson (http://www.tree.caltech.edu). Pairs of overgo primers 1 µl each (10 pmol) were added to 3.5 µl H_2O and denatured at 80°C for 5 min, followed by 37°C for 10 min and cooled on ice. Heat-denatured overgo primer pairs were mixed with 0.5 µl BSA (2 mg/µl), 2 µl OLB (oligo labeling buffer without dATP, dCTP, and random hexamers), 1 µl Klenow fragment (2 U/µl), 0.5 µl [α-^{32}P]dATP (3,000 Ci/mmol) and 0.5 µl [α-^{32}P]dCTP (3,000 Ci/mmol). Then, the 10-µl reaction mixture was incubated for 1 h at room temperature, followed by addition of 40 µl TE to stop the reaction. All overgos were labeled individually, combined according to pools required and then run through a Sephadex G-50 column (Amersham) to remove unincorporated radioactive nucleotides. The labeled overgos were denatured at 95°C for 5 min before using for hybridization. To assist in accurately localizing each positive clone on the high-density clone filters, approximately 0.1 µl (~0.02 ng) of pECBAC1 and/or pCLD04541 vector DNA was labeled using the Rediprime II Random prime labeling system (Amersham Biosciences, RPN1633), denatured at 95°C for 5 min and applied to the BAC and/or BIBAC library filter hybridization solution. Three barley chloroplast DNA probes, *ndh*A, *rbc*L, and *psb*A, and one wheat mitochondrial DNA probe, *cox*II, were labeled by using the same random priming method, and used for screening the BAC and BIBAC libraries to determine the genome origin of the clones.

Identification of positive clones by two-step screening

The libraries were screened in two steps. In the first step, the pooled probe of all overgos of the RFLP markers was used to hybridize the high-density clone filters in a single hybridization. The positive clones were re-arrayed in 384-well microtiter plates, and used to produce secondary filters. In the second step, the positive clones spotted on the secondary filters were further verified and sorted according to their contents of individual overgos by a two-dimensional (row pools and column pools) overgo pooling hybridization strategy. Each overgo was present in a fixed row number and a fixed column number. Clones hybridized with both a row pool probe and a column pool probe were assigned to the positive clones of the overgo residing at the intersection between the row and column pools (Cai et al. 1998; Romanov et al. 2003).

High-density clone filters representing a subset (5.1×) of the libraries were screened with the pooled probes of 36 overgos. The filters were prehybridized in the hybridization buffer at 60°C for 4 h. The labeled, denatured probes were added and allowed to hybridize overnight at 60°C. After hybridization, the filters were washed in a series of buffers with increasing stringency: 2× SSC and 0.1% SDS at room temperature for 30 min, 1.5× SSC and 0.1% SDS at 60°C for 30 min, and 0.5× SSC and 0.1% SDS at 60°C for 30 min (Sambrook et al. 1989). The filters were sealed in plastic sheets, exposed overnight, and then scanned with a Typhoon 9410 Variable mode imager (Amersham).

Results

Construction and characterization of the BAC and BIBAC libraries

To accommodate different research purposes of sunflower genomics, we constructed a BAC library in pECBAC1 with *Bam*HI and a plant-transformation-competent BIBAC library in pCLD04541 with *Hin*dIII from cultivar HA 89 (Table 1). The BAC library contained 107,136 clones, and was arrayed in two hundred and seventy-nine 384-well microtiters. From the library, 225 clones were randomly selected and analyzed by *Not*I digestion and pulsed-field gel electrophoresis. One (0.44%) of the 225 BAC clones had no insert and the others had a mean insert size of 140 kb. The BIBAC library contained 84,864 clones and was arrayed in 221 microtiters. Similarly, 196 clones were randomly selected from the BIBAC library and analyzed by *Not*I digestion and pulsed-field gel electrophoresis. The mean insert size of the clones was 137 kb (Fig. 1), and all of the 196 clones contained inserts, indicating that insert-empty clones were rare in the BIBAC library. The insert size distribution of 421 BAC and BIBAC clones are illustrated in Fig. 2, with a majority of the clones carrying inserts ranging from 120 to 150 kb. The average insert size of BAC clones was slightly larger than that of the BIBAC

clones, but the difference was not significant. The number of *Not*I sites in the inserts of the clones ranged from 0 to 1 (Fig. 1); most clones had no *Not*I site in their inserts, as indicated by a single insert band on pulsed-field gels. However, a few clones had one *Not*I site in their inserts, evidence of two insert bands on the gels.

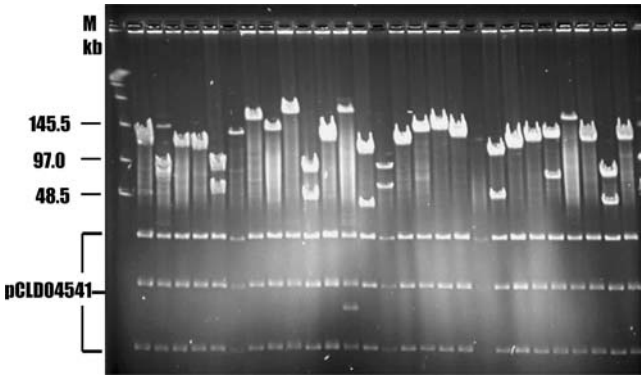

Fig. 1 BIBAC clones randomly selected from the sunflower BIBAC libraries. DNA was isolated, digested with *Not*I, separated by PFGE (5–15 s switch time, 6 V/cm, 120° angle, 12.5°C, 16 h in 0.5× TBE buffer), and stained with ethidium bromide. The letter "M" indicates lambda ladder

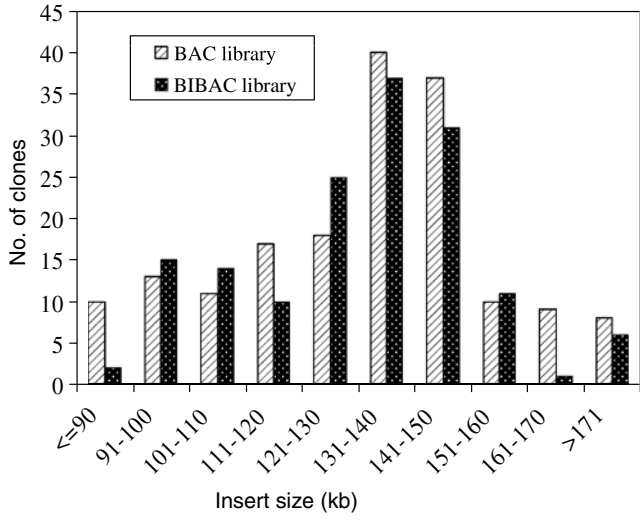

Fig. 2 Insert size distributions of 421 clones randomly selected from the *Bam*HI BAC library and *Hin*dIII BIBAC library

Table 1 Characteristics of the BAC and BIBAC libraries of sunflower cultivar HA 89

Features	BAC library	BIBAC library	Combined
Cloning vector	pECBAC1	pCLD04541	2
Restriction enzyme	*Bam*HI	*Hin*dIII	2
No. of clones arrayed	107,136	84,864	192,000
No. of 384-well microtiters	279	221	500
Average insert size (kb)	140	137	139
Insert-empty clones (%)	0.44%	0.0	<0.5%
cpDNA clones (%)	2.10%	2.59%	2.35%
mtDNA clones (%)	0.03%	0.04%	0.04%
Genome equivalents	5.0×	3.9×	8.9×

To estimate the proportion of clones that originated from organellar DNA, one high-density clone filter of each library containing 18,432 clones was screened with a mixture of three barley chloroplast genome-specific genes, *ndh*A, *rbc*L, and *psb*A, and one wheat mitochondrial gene, *cox*II, as probes, respectively. The results indicated that 2.10 and 2.59% of the clones contained chloroplast DNA (cpDNA), and 0.03 and 0.04% contained mitochondrial DNA (mtDNA) for the BAC and BIBAC libraries, respectively. Together, the clones of the libraries originating from the chloroplast and mitochondrial genomes were 2.35 and 0.04%, respectively. Given that sunflower has a haploid genome of 3,000 Mb, the BAC and BIBAC libraries represent approximately 5.0× and 3.9× of the sunflower genome, with the probability of obtaining a clone of interest from each library 99.3 and 98.0%, respectively (Ren et al. 2005). The combined two libraries represent 8.9× of the genome with the probability of obtaining a clone of interest from the combined libraries being greater than 99%.

Identification of a set of linkage group-specific clones by overgo hybridization

To further evaluate the libraries and isolate BACs and/or BIBACs specific for each chromosome of sunflower ($x = 17$), we screened the libraries with overgos designed from the sequences of previously mapped RFLP markers (Jan et al. 1998). The RFLP linkage map consisted of 20 linkage groups, including three questionable groups. One to three single- or low-copy RFLP markers were selected from each of 19 linkage groups (the smallest one has no sequenced marker) to design the overgo primers. A total of 36 overgos representing 19 linkage groups were designed, synthesized and used in the library screening.

We screened six high-density clone filters, three from each library, and each filter contained 18,432 double-spotted clones. This subset library represented a 5.1× genome coverage and contained approximately 110,592 clones. The clones were screened in two steps (Fig. 3). In the first step, all 36 overgos were individually labeled and pooled, and used a single probe to hybridize the six high-density clone filters. A total of 206 positive clones were identified (Fig. 3a). In the second step, the 36 overgos were aligned into six row pools and six column pools, with each pool containing six overgos. The 206 positive clones identified in the first step were double-spotted on nylon filters and hybridized with each of the six row pools and six column pools, respectively (Fig. 3b). The positive clones shared between the row and column pool probes were assigned to be the positive clones of the overgo at the intersection between the row and column pools. Of the 206 positive clones identified at the first step, 195 were confirmed in the second step.

Furthermore, the addresses of all the 195 BACs or BIBACs were determined and assigned to 19 linkage groups (Table 2). Of the 36 overgos analyzed, 33 (92%) hit at least one positive clone, and three (8%), including 21D2, 6B3 and 15D4, failed to hit any clone. Two overgos, 15D2 and 14E5, hit the same clones (63A12, 160C8, 395G7, 466I23, and 480H15) even though they were from different linkage groups. We analyzed the sequences of the two overgos and found that the two overgos share a 13-nucleotide sequence. Screening of a subset (5.1×) of the BAC and BIBAC libraries with 36 overgo probes identified 195 positive clones, averaging 5.4 hits per overgo. Considering the fact that only 57.6% (5.1×) of the clones of the libraries were screened in this experiment, the genome coverage (10.3×) of the libraries estimated by the overgo hybridization is consistent with the 8.9× genome equivalents calculated by using the average insert sizes of the libraries and the sunflower genome size (3,000 Mb/1C).

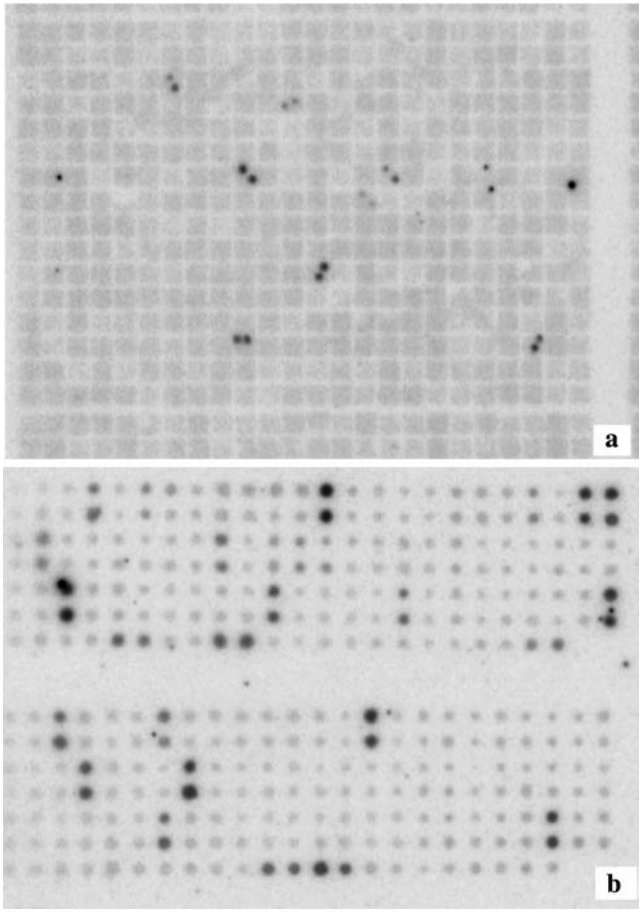

Fig. 3 **a** A panel of a six-panel high-density clone filter screened with pooled 36 overgos. **b** Identification of positive clones for each overgo. The positive clones identified in (**a**) were re-arrayed, double-spotted on new filters and re-hybridized with each column and row pool of the 36 overgos. The figure shows two filters of the BIBAC clones hybridized with two different column pools: first pool: 1E6, 7F3, 9F2, 15C2, 2B4 and 15A6 (*top panel*, **b**); and second pool: 7C1; 20B2, 6E6, 21D2, 15E3, and 6B3 (*lower panel*, **b**)

A significant difference in the number of hits per overgo was also observed between BAC and BIBAC libraries. Of the 33 overgos that identified positive clones (Table 2), 21 (63.6%) hit both BACs and BIBACs, but two (6.1%) hit only the BACs, and 10 (30.3%) hit only BIBACs. In other words, the 33 overgos identified 76 positive BACs and 119 positive BIBACs even though the genome coverage (2.58×) of BACs was the same as that of BIBACs (2.54×), suggesting that the BIBAC library had a better realized genome coverage than the BAC library. The biased distribution of the positive clones in the two libraries further confirmed that the complementary effect of the libraries provides the more comprehensive genome coverage.

Discussion

Preparation of high-quality megabase genomic DNA and determination of optimal partial digestion conditions are crucial for quality BAC library construction. Size selection of DNA partially digested in LMP agarose plugs was based on pulsed-field electrophoresis on an agarose gel. Frequently, three fractions of the gel containing DNA fragments in ranges of 100–200, 200–300, and 300–400 kb were selected in the first size selection. This was followed by a second size selection for further removal of smaller DNA fragments (Zhang 2000; Vilarinhos et al. 2003). Yüksel and Paterson (2005) showed that BACs that had desirable large insert sizes and high transformation efficiency were frequently obtained from the middle fraction of the gel, whereas the smaller and larger size fractions often produced either smaller insert clones with higher transformation efficiency (for the smaller size fraction), or higher percentages of insert-empty clones (no insert) with lower transformation efficiency (for the larger size fraction). In our procedure, we performed only one size selection and selected the fraction of 150–250 kb for BAC and BIBAC cloning. These modifications not only assured sufficient large-insert clones, but also maintained higher transformation efficiency by reducing damage to clonable DNA fragments that would occur during a second round of size-selection.

We constructed a BAC library and a plant-transformation-competent BIBAC library for sunflower. These BAC and BIBAC libraries have several advantages over the sunflower BAC libraries previously reported (Gentzbittel et al. 2002; Özdemir et al. 2004) for sunflower. First, the new libraries (~139 kb) have a much larger average insert size than the existing sunflower libraries (60–80 kb). It has been documented that the larger insert size enhances their utility in genomics research, including positional cloning and genome physical mapping (Zhang et al. 1996b; Ren et al. 2005). This is especially important for species with large genomes such as sunflower. Second, the new libraries have a deeper genome coverage (8.9×) than that of the combined existing BAC libraries (6–7×) (Gentzbittel et al.

Table 2 Positive clones identified by screening a subset (5.1×) of the sunflower BAC and BIBAC libraries with 36 overgo primers designed from single- or low-copy RFLP markers

Linkage group	Overgos (RFLP markers)	Number of hits	Positive clones	
			BAC library (*Bam*HI)	BIBAC library (*Hin*dIII)
1	20A5	3	176D13	374I4, 386G6
2	1E6	1		438A20
	7F3	3		408N21, 375M11, 389P23
	5E4	2		387P13, 455J18
3	9F2	5	59A24, 85F5, 95G15	405C18, 412G14
	4B6	4		382L5, 464F20, 479B11, 479C23
4	15C2	5	175B15, 185H20	372N14, 394G13[a], 493E17[a]
	14A2	18	61N8, 63H22, 75N23, 85K23, 113F3, 113H4, 113K6, 117I4, 120O14, 157P17, 183J8	387C9, 389F19, 415L4, 443C19, 479N10, 480G10, 510B10
5	2B4	3		459O10, 477M11, 481K13
	2D4	6	73I23, 155P12, 180O2, 187P23	379K19, 477D22
6	15A6	6	177F3	394G13[a], 410H1, 484D12, 493E17[a], 510G12
	15D2[b]	7	63A12[a], 126N9, 141K9, 160C8[a]	395G7[a], 466I23[a], 480H15[a]
7	7C1	5	115K11	384K22, 391A6, 391J5, 475D1
	21E6	3	160C8[a]	395G7[a], 429J21
8	2E2	1	104I23	
	20F1	5	103H6, 124A11	381E4, 403O10, 470I10
9	20B2	5	75A6, 92I11, 92K9	385L10, 503I7
	1C5	4	60L23, 183P19, 184P8	398F18
10	4D1	4		381J20, 433N17, 437K12, 440B2
	7D5	9	124J4, 135J2, 143N10, 207E1	382G9, 382M4, 403G22, 480D18, 509G6
11	10C4	16	60O16, 90I16, 150I16, 150N10	383C12, 388I20, 404J3, 415O6, 422H19, 437I16, 439M12, 444J19, 457L19, 458B16, 477G6, 489M22
	6E6	1	115B2	
12	10D6	4		384O18, 389J8, 438D10, 458N24
	21D2	0		
13	15E3	11	67L19, 81K21, 92L2, 112C20	372M22, 376A19, 397M4, 402M16, 447G18, 480G16, 498N8
	11A6	4		380F19, 407K6, 421M12, 509I15
14	8E4	5	62M10, 163M16	422O1, 426G11, 490B11
	6B3	0		
15	8C4b	6		367P3, 382M16, 437F7, 445H4, 466O6, 466O7
	9D1	1		401C5
	15D4	0		
16	8A1	10	61L11, 76D23, 78G18, 84A4, 155C8	425P7, 446E13, 453H24, 460G12, 505K20
17	10B5a	8	75B15[a], 110G13, 159L12[a], 159N24[a], 183A20[a]	368F18[a], 390A9, 428O6
18	13E4	14	84K7, 88C11, 130H14, 204N17	369D23, 384J13, 420L5, 421E12, 426F15, 435K3, 467G11, 474E15, 481N22, 483C7
	14E5[b]	6	63A12[a], 159I12, 160C8[a]	395G7[a], 466I23[a], 480H15[a]
19	21F1	10	75B15[a], 151G15, 159L12[a], 159N24[a], 160C8[a], 178O1, 183A20[a]	368F18[a], 395G7[a], 477M12
Total	36	195	76	119

[a]Clones hit by different, unlinked overgos
[b]Overgos15D2 and 14E5 share a 13-bp sequence

2002; Özdemir et al. 2004), which is essential for comprehensive genome research. Moreover, the two previous BAC libraries were constructed with *Hin*dIII in the pBeloBAC11 vector (Gentzbittel et al. 2002; Özdemir et al. 2004), which may bias distribution of their clones along the genome (Zhang et al. 1996a; Tao et al. 2002; Chen et al. 2004; Wu et al. 2004b). In contrast, the new libraries were constructed with two enzymes (*Hin*dIII and *Bam*HI) that are complementary in the nucleotide content of restriction sites and two vector systems (pECBAC1-F plasmid-based and pCLD04541-P1 plasmid-based); therefore, their clones should be better distributed throughout the genome than those of the existing libraries. Finally, the BIBAC library reported here represents the first plant-transformation-competent BIBAC library for sunflower. Because BIBAC clones are competent for direct transformation by the *Agrobacterium*-mediated transformation technique (Hamilton et al. 1996, 1999), the sunflower BIBAC library will facilitate gene and QTL positional cloning, and functional analysis of sunflower genomic sequences once the large DNA fragment transformation techniques are established in sunflower.

Owing to the high throughput over conventional probe hybridization, overgo hybridization has become the method of choice for screening BAC libraries,

particularly when a large number of probes are to be assigned in a library. Using this strategy, we were able to identify the positive clones of 33 of the 36 overgos analyzed. While some overgos (e.g. 10C4 and 14A2) hit many more clones than the expected 5.1 clones, probably due to their multiple loci in the genome, most overgos hit approximately the expected number of positive clones. This suggests that most of the positive clones identified with the overgos could be locus- or chromosome-specific, even though further investigation is needed to verify this conclusion. Instead of direct two- or more dimensional pooling hybridization strategies (Cai et al. 1998; Gardiner et al. 2004), we chose a procedure of two-step library screening by use of overgo hybridizations (Han et al. 2000). The first step was used to identify the positive clones of all overgo probes from the high-density clone filters, while the second step, a typical two-dimensional overgo pooling strategy, was used to determine the overgo-clone relationships. This strategy avoids repeated row and column pool probe hybridizations on a large number of high-density clone filters and further verifies the positive clones identified in the first step by the second step row and column pool hybridizations, and reduces the number of ambiguous and false positive clones, as indicated in this study (11 false positive clones).

Of the 36 overgos used in the library screening, 33 (92%) hit at least one positive clone. This success rate is comparable to those previously reported (Cai et al. 1998; Han et al. 2000; Gardiner et al. 2004), though it may be significantly affected by the genome coverage of target libraries and the overgo pool sizes. The failure of three overgos in identifying positive clones from the libraries may be due to the use of a subset (5.1×) of the libraries, inaccurate sequences of the target RFLPs, improper overgo design, failed labeling, or improper hybridization stringency for every overgo in the pool. For example, the sequences of some RFLP markers could not be used to design overgos or must be done so in a relaxed design criterion due to the abundance of repetitive sequences contained. Similar to Romanov et al. (2003), some clones in our libraries were hit by two unlinked overgos. For instance, the clone 394G13 was simultaneously hit by overgo 15C2 from linkage group 4 and 15A6 from linkage group 6 (Table 2). This suggests that these clones might represent duplicated regions of the genome, or due to occasional errors in the re-arraying and array fabrication of the positive clones identified in the first step of the library screening. This could be verified by further analysis of the clones.

The 195 positive clones identified by using the linkage group-specific overgo probes represent 19 of the 20 linkage groups of the sunflower genetic map (Jan et al. 1998). Since the genome of sunflower consists of 17 chromosomes ($x = 17$), these linkage group-specific BAC/BIBAC clones will provide useful tools for sunflower chromosome identification and manipulation. We are currently employing the BAC and BIBAC clones as probes in fluorescence in situ hybridization to identify individual chromosomes in the sunflower (Feng et al. 2005). This will allow identification of a set of sunflower trisomics and the relation of the linkage groups with individual chromosomes, and facilitate the construction of a physical map by using these directly observable cytogenetic markers.

Acknowledgements We thank Wei Sun, Lisa Brown, and Leonard Cook for assisting in the array of the libraries, Pilar Rojas and Junfang Chen for their kind help in the lab, and L. G. Campbell and G. J. Seiler for critically reading the manuscript. We are also grateful to Justin Faris for the use of specialized equipment, and to A. A. Schneiter and staff at the Department of Plant Sciences, North Dakota State University, for assistance in administering this grant. Finally, thanks are given to R. Kesseli for sequencing the RFLP markers used in this study. This project was supported by the U.S. Department of Agriculture CRIS project 5442–21000-027-02.

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