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ORIGINAL PAPER

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Construction and characterization of a soybean yeast artificial chromosome library and identification of clones for the *Rps6* region

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Abstract We report the construction and characterization of the first soybean yeast artificial chromosome (YAC) library using high-molecular weight DNA isolated from leaf nuclei of the cultivar Conrad 94 that carries *Phytophthora* resistance genes *Rps1*-k and *Rps6*. The quality of this library has been evaluated through analysis of 393 randomly selected YAC clones. The library consists of 36,864 clones, of which ~19,956 carry single soybean YACs with an average size of about 285 kb. The library represents approximately five soybean genome equivalents. The probability of finding any soybean sequences from this library is about 0.99. The library was screened for 43 SSR markers representing the whole soybean genome. We were able to identify positive YAC pools for 95% of the SSR markers. Two YAC clones carrying molecular markers linked to the *Rps6* gene were identified. The YAC library reported here would be a useful resource for map-based cloning of agronomically important soybean genes and also to complement the effort towards construction of the physical map for the soybean genome.

Keywords Soybean · YAC library · Positional cloning · Resistance genes

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Introduction

Large insert genomic libraries such as yeast artificial chromosome (YAC) libraries have been constructed for many important plant species like *Arabidopsis* (Grill and Somerville 1991), rice (Umehara et al. 1995), corn (Edwards et al. 1992), barley (Kleine et al.1993), tomato (Martin et al. 1992), potato (Leister et al. 1997) and pepper (Tai and Staskawicz 2000). Such libraries have facilitated positional cloning of genes and genomic regions in many of these species (Martin et al. 1993; Bent et al. 1994; Yoshimura et al. 1996; Brommonschenkel and Tanksley 1997; Tai et al. 1999; Teraishi et al. 2001), physical genome mapping (Kurata et al. 1997; Sato et al. 1998; Saji et al. 2001), analysis of repetitive sequences (Dunford and Rogner 1991; Schmidt et al. 1994), and studying the relationship between genetic and physical distances (Civardi et al. 1994). The main advantages of YACs over prokaryotic-based cloning systems are its large insert size (100–1000 kb), and ability to maintain sequences that are unstable and not well represented in prokaryotic cloning system. Nearly all DNA sequences are clonable in YACs (Schlessinger et al. 1991), as yeast can tolerate several kinds of DNA structures that may not be properly propagated in *Escherichia coli* (Burke et al. 1987). The power and utility of YAC libraries is particularly evident from positional cloning of agronomically important genes with a reduced number of chromosomal walking steps from crop species that have large and complex genomes (e.g. Martin et al. 1993; Tai et al. 1999).

Soybean, one of the most important crops in North America, is a diploidized tetraploid (Hadley and Hymowitz 1973; Shoemaker et al. 1996). Several soybean BAC libraries have been constructed (Marek and Shoemaker 1997; Danesh et al. 1998; Salimath and Bhattacharyya 1999; Tomkins et al. 1999; Meksem et al. 2001) and are being used for physical mapping of the soybean genome (Marek et al. 2001; Meksem et al. 2001). The average insert sizes in these libraries are reported to be about 100–150 kb. To date no usable soybean YAC

library is available. Zhu et al. (1996) reported a partial soybean YAC library representing approximately 0.25 equivalent of the soybean genome. Therefore, the utility of this library is very limited for positional gene cloning and physical mapping. The main objectives of the present experiments were to generate and characterize a soybean YAC library, and to evaluate the utility of this library for map-based cloning of the agronomically important *Phytophthora* resistance gene *Rps6*.

Materials and methods

Plant material and yeast strain

Soybean cultivar Conrad 94 carrying the resistance genes *Rps1*-k and *Rps6* conferring resistance to *Phytophthora sojae* was used as a source of DNA for the library construction. Seeds were grown in peat moss under suitable growing conditions (Bhattacharyya and Ward 1986). Young unifoliate leaves from 10-day-old seedlings and subsequently fully expanded tri-foliates from the same seedlings were used for nuclei isolation. *Saccharomyces cerevisiae* strain YPH252 (*Mat*$\propto$*, ura3–52, lys2–801*^{amber}*, ade2–101*^{ochre}*, trp1-*Δ*1, his3-*Δ*200, leu2-*Δ*1*) obtained from ATTCC was used for preparation of spheroplasts.

Pulsed field gel electrophoresis

High molecular weight (HMW) soybean and yeast chromosomal DNAs were analyzed by contour-clamped homogeneous electrophoresis (CHEF) using a CHEF DR II apparatus (Bio-Rad, Richmond, Calif.). The electrophoresis was carried out at 200 volts (6 v/cm) with 50- to 90-s ramped switch time for 22 h at 14°C.

Isolation of HMW DNA from soybean leaf nuclei

The HMW DNA was prepared from leaf nuclei isolated by following the protocol of Salimath and Bhattacharyya (1999). Nuclei were pelleted by centrifuging in a Sorval table-top centrifuge at 1,920 *g*. Solidified plugs were processed according to Ganal and Tanksley (1989). The PMSF was removed by dialyzing plugs thoroughly in at least 10 volumes of T.E. (10 mM Tris, 1 mM EDTA, pH 8) at 50°C for 1–2 h 2 or 3 times.

Fragmentation of HMW soybean DNA and preparation of vector

Embedded HMW DNA was partially digested by employing a competition assay of *Eco*RI and *Eco*RI methylase described by Bonnema et al. (1996). The ratio of *Eco*RI to *Eco*RI methylase that generated restriction fragments of about 300–800 kb was chosen for preparative digestion of HMW DNA. Partially digested HMW DNA was electrophoresed in a 1% Seaplaque GTG agarose CHEF gel to compress DNA fragments of >300 kb into a zone of limited mobility (Tai and Staskawicz 2000). The pJS97/98 YAC vector system (Shero et al. 1991) was used for the generation of the YAC library. YAC vector arms were prepared from the purified circular DNA (Tai and Staskawicz 2000). The DNA mixture was digested with *Eco*RI. Ligation and subsequent size selection was done according to Tai and Staskawicz (2000).

Preparation and transformation of yeast spheroplasts with YAC DNA

Spheroplasts were prepared and transformed according to Tai and Staskawicz (2000). Transformants were transferred to DOB-UT agar plates (2% dextrose, 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 0.72 g/l UraTrp dropout powder, 2.2% Noble agar).

Analysis of individual YAC transformants

Yeast chromosomal DNA plugs were prepared using a miniprep procedure (Burke 1989) and were analyzed by CHEF gel electrophoresis. The CHEF gels were blotted onto Zeta-Probe GT nylon membrane by following the procedure described in the Bio-Rad CHEF-DR II pulse field gel electrophoresis instruction manual. The blot was hybridized to ^{32}P-labeled soybean genomic DNA or vector sequences by using the formamide hybridization buffer (Sambrook et al. 1989).

Screening of library

Yeast chromosomal DNA preparation and screening of the library was carried out according to Green and Olson (1990). Ninety-six clones were mixed and grown as a single primary pool for DNA preparation. Equal quantities of DNA samples from four primary pools were mixed to form a superpool representing 384 YAC clones of a microtiter plate. Screening was first carried out on superpools. About 100 ng DNA was used in individual PCR reactions. The sequences of soybean SSR primers used in this investigation can be obtained from soybase (http://129.186.26.94/ssr.html). Primer pairs (1) 160N2UF2 (5′-GCCTCTCCTTTGAAGTTG-3′) and SNPFL (5′-CTCGTATAGCTTAAAGACACATTTCCGTC-3′) for the 160N2U marker, and (2) 44C18UF (5′-AATATCATTGTCCTCCATATTTTTATCC-3′) and 44C18UR (5′-CTGACTATCAACAGAGTTTATGGAACAT-3′) for 44C18U were used in screening the library. These two markers are from the *Rps6* region. The PCR conditions used were 94°C for 3 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for an additional 8 min. Four primary pools that constitute the positive superpools were then rescreened to identify the positive primary pool. The positive YAC clone from the primary pool was identified by colony hybridization of 96 YAC clones (Brownstein et al. 1989).

The primer pair (5′-CCAGTCTTGATCGTTACAAAG-3′ and 5′-AGTCATGCATTACGATCGGAA-3′) specific to the large subunit of ribulose-bisphosphate carboxylase/oxygenase was used to screen the superpools by PCR in order to determine the extent of chloroplast DNA contamination in the library. To determine the extent of mitochondrial sequences in the library the superpools were screened by PCR using the primer pair (5′-CCAAGCAATGCCCAAAAGTC-3′ and 5′-TCGTCTGCACTGGTTTGTAC-3′), which is specific to the mitochondrial complex I subunit of NADH-dehydrogenase (Leister et al. 1997). The frequency of chloroplast or mitochondrial sequences in the YAC library was estimated according to Leister et al. (1997).

Results and discussion

The soybean YAC library

The soybean YAC library was generated using partially digested size-fractionated (>300 kb) DNA. The size-selected insert DNA was ligated with YAC vector arms. A second size selection (>300 kb) of the ligation products was performed before transformation to improve the size of YAC clones. The spheroplast transformation technique was standardized to obtain transformation efficiency of at least 10^6 transformants per μg of circular plasmid vector DNA. Yeast spheroplasts were transformed with high molecular weight ligated YAC DNA molecules. In

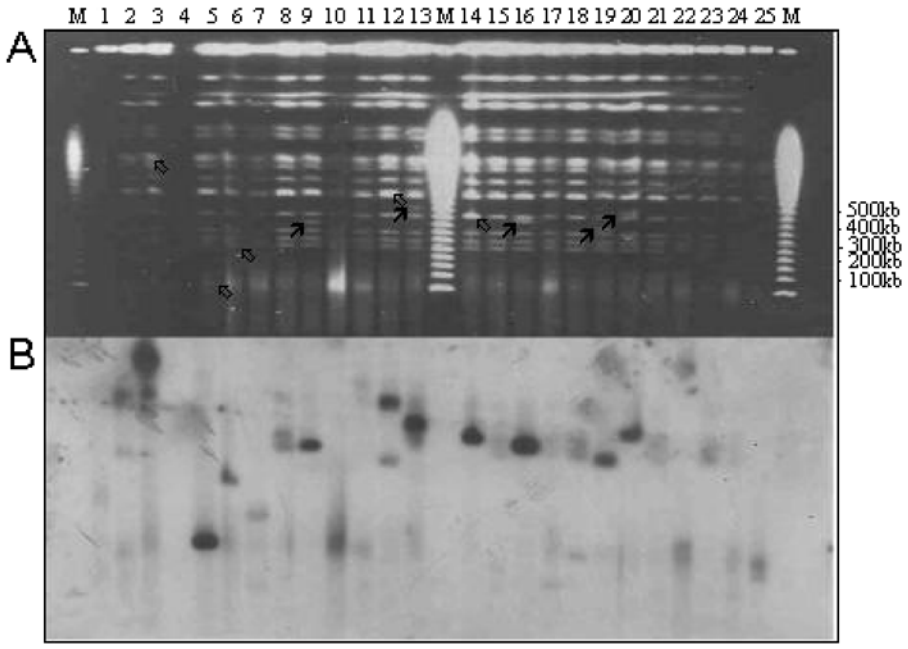

Fig. 1A, B DNA-gel blot analysis of randomly selected yeast transformants. **A** CHEF gel stained with ethidium bromide (*above*). *Lanes 1–25* Randomly chosen YAC transformants; *lane M*, 50-kb lambda ladder marker. YACs which are visible as an extra DNA band are marked with filled arrows pointing upward to right while YACs superimposed by yeast chromosomes are shown by open arrows pointing upward to left. **B** Gel blot hybridized to radiolabeled soybean genomic DNA

routine transformation experiments, yeast spheroplast transformation always resulted in 10^6 to 10^7 transformants per μg of the circular vector pJS97 DNA. In contrast, transformation efficiencies with ligated YAC DNA ranged from about 50 to 2,500 transformants per μg of ligated DNA. Transformed spheroplasts were plated directly onto media lacking both uracil and tryptophan, in order to select transformants containing both vector arms. This double selection increases the frequency of transformants carrying an intact YAC. The library consisted of 36,864 clones obtained from transformation of 21 ligations. Quality of clones obtained from each ligation was monitored by analyzing a random sample of about 9–28 YAC clones for size and extent of hybridization to soybean genomic DNA and vector sequences. Ligations producing soybean YACs with average size bigger than 150 kb were only considered for the library construction. Most YAC libraries reported in the past have been constructed using the pYAC4 vector system (Burke et al. 1987). However, several other YAC cloning vectors are also available (Smith et al. 1990; Shero et al. 1991). We used the pJS97/98 cloning system that was previously used by Tai and Staskawicz (2000). This vector system allows the retrieval of both ends of YAC clones directly from *E. coli* because centric and acentric vector arms were constructed as separate plasmids and both ends of YACs carry the origin of DNA replication for *E. coli* (Shero et al. 1991).

Characterization of the library

The library was constructed from 21 independent ligations. A total of 393 random clones were analyzed from 21 sub-libraries generated from these ligations. The average insert sizes of these sub-libraries varied from 150 to 430 kb. A representative photograph of a gel and its corresponding autoradiogram showing soybean YACs is presented in Fig. 1. The YACs are not always visible as additional chromosomes, because some of the YACs are superimposed by yeast chromosomes. It was calculated that about 55% of the transformants contained single soybean YACs. The remaining transformants belonged to the following classes: (1) one class (about 9%) consisted of transformants with no YACs; this suggests that these clones were false positive. (2) Transformants in the second category (about 4.5%) displayed hybridization patterns made up of more than two bands. These YACs represent either a mixture of YAC clones and/or clones that had undergone chromosomal recombination. (3) A large number of transformants (about 31.5%) showed a single band of about 550 kb hybridized to vector sequences, but not to the soybean genomic DNA. Most likely, insertion of the vector into a specific yeast chromosomal region through illegitimate recombination resulted in this class of clones. YACs from classes 1 and 3 do not contain soybean DNA and are ineffective. For this reason these two classes of transformants were excluded from estimation of the genome coverage for the library. Similarly, class 2 clones were also not usable and not considered for average size estimation. The frequency distribution of insert sizes among 196 YAC clones carrying single soybean DNA inserts is shown in Fig. 2. The size of YAC clones varies from 50 to 900 kb and about 50% of the clones are in the range of 300 to 500 kb. The average insert size of these clones in the library is ~285 kb.

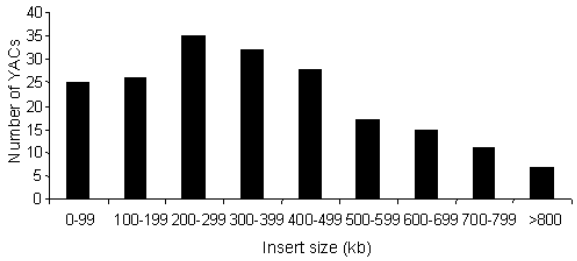

Fig. 2 Size distribution of randomly selected YAC clones. Randomly selected YAC clones from each ligation reaction were sized in CHEF gels by carrying out DNA-blot hybridization with soybean genomic DNA as the probe

Screening of the library for SSR markers

In general SSR markers are mapped to single loci of the soybean genome. We selected 43 SSR markers that mapped to single loci. These markers represent all 20 chromosomes. At least two SSR markers were selected from each chromosome. These markers were used to screen 96 superpools of the library. A total of 168 positive YAC superpools, with an average of at least 3.9 superpools/marker, were identified (Table 1). Each of the positive superpools consisted of 384 clones, so it is possible that some of the positive superpools may contain more than one positive clone. Maximum number of clones identified by an SSR marker was 13 and minimum was 0. Two SSR markers (Satt195 and Satt201) identified no positive clones from the library. We identified positive clones for 95% of the SSR markers (Table 1). The

Table 1 YAC superpools containing SSR markers from the whole soybean genome

Sl. no.	SSR[a]	MLG[b]	Positive superpool	No. of positive superpools
1	Satt073	A1	90, 91	2
2	Satt200	A1	18, 25, 26, 27, 28, 35, 38, 62, 70, 78, 83, 84, 85	13
3	Satt119	A2	20, 59	2
4	Satt089	A2	38	1
5	Satt298	B1	61, 90, 94	3
6	Satt332	B1	16, 32, 44, 52, 62, 71, 80	7
7	Sct_034	B2	73	1
8	Satt467	B2	46, 53, 62, 77	4
9	Satt136	C1	7, 23, 27, 28, 30, 31, 45	7
10	Satt195	C1	0	0
11	Satt079	C2	21, 89	2
12	Satt202	C2	5, 17, 38, 61, 73, 75, 77, 80, 85, 93	10
13	Satt032	D1a+Q	80	1
14	Satt147	D1a+Q	5, 14, 21, 42, 69	5
15	Satt041	D1b+W	3, 8, 13, 16, 44, 57	6
16	Satt266	D1b+W	40, 56	2
17	Satt208	D2	71, 85	2
18	Satt226	D2	16, 24, 26, 63	4
19	Satt117	E	10, 25, 43, 69	4
20	Satt213	E	8	1
21	Satt145	F	52	1
22	Satt193	F	17, 44, 45, 57, 60, 76, 93	7
23	Satt130	G	3, 4, 11, 19, 42, 74, 77	7
24	Satt217	G	19, 25, 85	3
25	Satt038	G	2, 43	2
26	Satt115	G	10, 34, 38, 40, 41, 49, 73	7
27	Satt163	G	10, 42, 62, 71, 77, 79, 87	7
28	Satt052	H	30, 39	2
29	Satt142	H	90	1
30	Satt292	I	90	1
31	Satt330	I	44	1
32	Satt132	J	3, 24, 26, 36, 38, 91	6
33	Satt215	J	5, 32, 39, 40, 65, 66, 74, 87	8
34	Satt381	K	14, 42, 60, 89	4
35	Satt178	K	3, 5, 11, 37, 75, 77, 79	7
36	Satt076	L	43	1
37	Satt229	L	37, 48, 88	3
38	Satt201	M	0	0
39	Satt210	M	11, 12, 29	3
40	Satt234	N	16, 24, 39, 63	4
41	Satt255	N	12, 38, 51, 59, 75	5
42	Satt094	O	23, 57, 72	3
43	Satt128	O	32, 38, 41, 42, 56, 75, 92, 94	8

[a] Information for SSR markers are available at http://129.186.26.94/ssr.html
[b] *MLG* Molecular linkage group (Cregan et al. 1999).

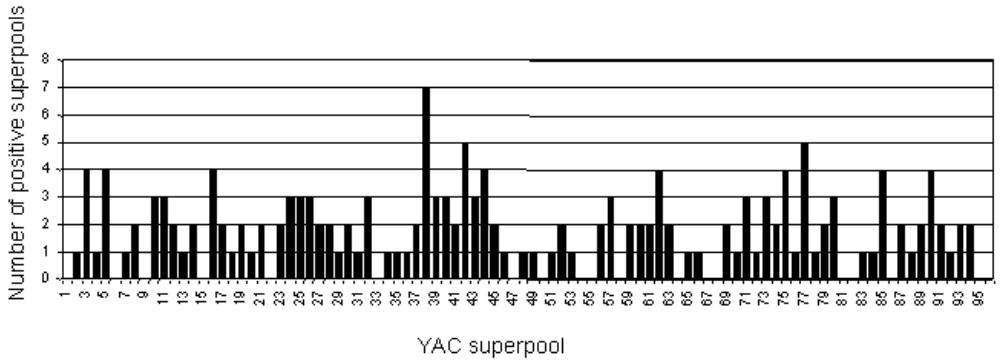

Fig. 3 Distribution of positive YAC superpools carrying SSR markers. The library carrying 96 superpools was screened for 43 SSR markers. Superpool 1 was obtained from the ligation 1, while superpool 96 was from ligation 21. Each *bar* represents the number of amplifications by one or more SSR markers in a specific superpool. The detail results are presented in Table 1

distribution of these positive YAC clones was nearly uniform throughout the library indicating that soybean YAC clones were generated from each of the 21 ligations (Fig. 3).

Screening of the library for chloroplast and mitochondrial sequences

The library was constructed from purified nuclear DNA. To estimate the extent of contaminating chloroplast and mitochondrial sequences the library was screened for the chloroplast-specific sequence the large subunit of ribulose-bisphosphate carboxylase/oxygenase and the mitochondrial-specific sequence the mitochondrial complex I subunit of NADH-dehydrogenase. The frequency of chloroplast- and mitochondrial-specific YAC clones were calculated according to Leister et al. (1997) and were 0.05% and 0.02%, respectively.

Identification of YAC clones containing sequences from the *Rps6* region and utility of the library

To test the utility of this YAC library for positional cloning of genes, we followed a PCR-based approach to identify YAC clones for the *Rps6* region. The 96 superpools of the library were screened for two markers, 160N2U and 44C18U, that cosegregate with the *Rps4/6* locus (D. Sandhu and M.K. Bhattacharyya, unpublished). One superpool was identified for each marker. Subsequent PCR analysis of four primary pools of each positive superpool and then colony hybridization of individual YAC clones from each positive primary pool resulted in identification of YAC clones soyYAC1E5 and soyYAC7M7 for the two *Rps6*-linked markers (Fig. 4). The approximate size of these two YAC clones are 350 kb and 700 kb, respectively.

A partial soybean YAC library consisting of less than 1,600 clones with average size of 175 kb has been reported earlier (Zhu et al. 1996). Based on a soybean haploid genome size of about 1.115×10^9 base pairs

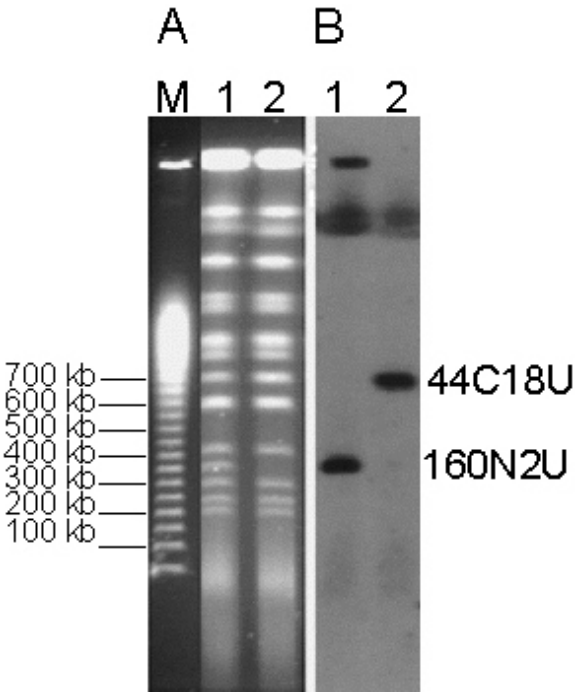

Fig. 4A, B DNA-gel blot analysis of YACs for the *Rps6* region. **A** CHEF gel of YACs from the *Rps6* region. **B** Gel blot hybridization with 160N2U and 44C18U probes. Lanes: *1* SoyYAC1E5 contains the marker 160N2U (~350 kb); *2* SoyYAC7M7 contains the marker 44C18U (~700 kb); *M* DNA marker 50-kb lambda ladder

(Arumuganathan and Earle 1991), the probability of identifying any sequences from this library is 0.22 (Clarke and Carbon 1976). The soybean YAC library we reported here consisted of 36,864 clones of which ~19,956 contain single soybean YACs with an average size of about 285 kb. The library should theoretically contain about five soybean genome equivalents. Thus, the probability of identifying any given sequence from this library is 0.99 (Clarke and Carbon 1976). Screening of this library for 43 SSR markers representing all 20 soybean chromosomes showed that the library contains 95% of these random sequences, a result very close to the theoretical probabil-

ity of 0.99 for finding any sequence from the library. We were able to identify two YAC clones for the *Rps6* region. This library can be used for positional cloning of the *Rps*6 gene and other agronomically important genes and can also be a useful resource for development of the soybean physical map.

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