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A bacterial artificial chromosome library for soybean and identification of clones near a major cyst nematode resistance gene

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Abstract We constructed a bacterial artificial chromosome (BAC) library for soybean (*Glycine max*) consisting of approximately 30 000 clones with an average insert size of 120 kilobase pairs. The library was successfully screened with restriction fragment length polymorphism (RFLP) and microsatellite markers tightly linked to a major resistance gene for the cyst nematode, *Heterodera glycines*. Since many soybean RFLPs hybridize to duplicate loci, BACs homologous to duplicate RFLP loci were distinguished by digestion with the restriction enzyme originally used to map the RFLP, followed by a comparison of the hybridizing fragments. Linkage mapping of BAC clones identified with markers linked to the cyst nematode resistance gene demonstrated that these clones were located at the expected chromosomal positions and that there were no indications of chimeras within the genomic inserts.

Key words Chromosome walking · Gene mapping · *Glycine max* · *Heterodera glycines* · High-molecular-weight DNA · Positional cloning

Introduction

Libraries consisting of large DNA inserts (i.e., 50 kilobase pairs or greater), such as yeast artificial chromosomes (Anand et al. 1989), P1 phage vectors (Sternberg 1990), and bacterial artificial chromosomes (BACs) (Shizuya et al. 1992), are useful in the physical mapping and positional cloning of plant genes (Martin et al. 1993; Bent et al. 1994; Grant et al. 1995; Song et al. 1995). Increasingly, plant scientists are focusing on BAC libraries because they are relatively simple to construct, inserts are easy to recover, and the frequency of rearrangement is low (Woo et al. 1994; Wang et al. 1995).

We are interested in genes involved in soybean-pathogen interactions, especially those that confer resistance to the soybean cyst nematode (SCN; *Heterodera glycines*). This organism is currently one of the most destructive soybean pests (Noel 1992). In previous work, we characterized genetic loci associated with quantitative resistance to SCN and found that a single major locus on molecular linkage group (MLG) 'G' was primarily responsible for the resistance phenotype (Concibido et al. 1997). In suitable mapping populations, this resistance gene could even be treated qualitatively (Concibido et al. 1996), opening the way to positional gene cloning. As a first step toward map-based cloning of this gene, we constructed a BAC library using DNA from the cyst nematode-resistant cultivar 'Faribault' (Orf and McDonald 1995). The usefulness of this library was demonstrated by screening it with several restriction fragment length polymorphism (RFLP) and microsatellite markers close to the SCN resistance gene, including two markers within 2 centimorgans (cM) of the gene.

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Materials and methods

Partial digestion and size-selection of high-molecular-weight DNA

Ten-day old soybean plants, cultivar Faribault, were held in complete darkness for 48 to 72 h. Unifoliate leaves were harvested and used for the preparation of high-molecular-weight DNA in agarose

plugs, as described by Danesh et al. (1995). Low-melting-point agarose (LMA) plugs containing 6.0 μg of DNA in a volume of 60 μl were incubated in a 140 μl *Eco*RI reaction buffer (4 mM spermidine, 50 mM NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.025% Triton X-100, 100 μg/ml bovine serum albumin) on ice for 60 min. The buffer was replaced with fresh buffer containing 3 units of *Eco*RI enzyme and incubated on ice for another 60 min. Partial digestion was accomplished by transferring plugs to a 37°C water bath for 5 min and stopped by adding 0.1 × vol of 0.5 M ethylene-diaminetetraacetic acid (EDTA), pH 8.0. Partially digested DNA was separated on a 1% LMA gel using a clamped homogeneous electric field (CHEF) (Chu et al. 1986) DR-II apparatus (Bio-Rad, Hercules, Calif., USA). The DNA was separated in TAE buffer (40 mM Tris-acetate, pH 8.0, 1.0 mM EDTA, pH 8.0) at 10°C, 6.0 V/cm with 90-s pulse time for 20 h. DNA fractions ranging from 225 to 285 kilobase pairs (kbp), 285–365 kbp and 365–450 kbp were excised from the LMA gel and used in ligation reactions.

BAC vector preparation

Vector pECSBAC4 (9.3 kbp) was kindly provided by Dr. Richard Michelmore, University of California, Davis, Calif., USA (Frijters et al. 1997). For preparation of the vector, the protocol of Woo et al. (1994) was used with the following modifications. After Qiagen (Chatworth, Calif., USA) Plasmid Maxi-Kit purification (using methods recommended by the manufacturer) and ethanol-precipitation, DNA was re-suspended in TA buffer (33 mM Tris-acetate, pH 7.8, 66 mM K-acetate, 10 mM Mg-acetate, 0.5 mM dithiolthreitol, 100 μg/ml bovine serum albumin). Plasmid DNA (10 μg) was digested to completion with *Eco*RI at 37°C for 4 h. After verification of plasmid digestion on an agarose gel, *Eco*RI enzyme was heat-inactivated by incubation at 65°C for 15 min. The DNA was then precipitated with ethanol and re-suspended in 60 μl of TA buffer plus 5 mM $CaCl_2$. The sample was divided into two 30-μl aliquots, one stored at −80°C for later use, while the other was de-phosphorylated using HK phosphatase (Epicenter Technologies, Madison, Wis.) (Woo et al. 1994). The extent and integrity of de-phosphorylated DNA was assayed by self-ligation. Ligation of 50 ng of de-phosphorylated plasmid DNA showed no detectable self-ligation, while a parallel reaction with ligase plus T4 polynucleotide kinase (New England Biolabs) resulted in self-ligation and circularization of the plasmid as monitored on an ethidium bromide-stained agarose gel. One-microgram aliquots of de-phosphorylated vector DNA were stored at −80°C until use.

BAC library construction

Partially *Eco*RI-digested soybean DNA in LMA was dialysed against TE (10 mM Tris, 1 mM EDTA, pH 8) on ice for 60 min and used in ligation reactions with *Eco*RI-digested and de-phosphorylated vector DNA following the protocol described in Woo et al. (1994). The ligation reaction was then subjected to drop dialysis (Marusyk and Sergeant 1980) for 2 h at room temperature. One microliter of the dialysed ligation reaction was used to transform 20 μl of *E. coli* Electro Max DH10B cells (Gibco-BRL, Gaithersburg, Md., USA) by electroporation (Sheng et al. 1995) using an ECM 600 electroporator machine (BTX, Inc., San Diego, Calif., USA) at the following settings (1-mm gap cuvette): choose-mode 2.5 kV, set-resistance 129 ohm, charging-voltage at 1.5 kV. Plating of transformed cells and selection of white (recombinant) colonies followed standard protocols (Woo et al. 1994; Wang et al. 1995). White recombinant BAC clones were picked manually and transferred to 384-well plates (Nalge Nunc International, Rochester, N.Y., USA) containing 70 μl of LB freezing medium with 12.5 μg/ml of chloramphenicol (CM) (Wang et al. 1995). The plates were wrapped in plastic and incubated at 37°C overnight or until sufficient bacterial growth was evident. Plates were then placed at −80°C for long-term storage. The library was replicated three times.

Characterization of the BAC library

For insert isolation, BAC clones were shaken in LB medium with CM (12.5 μg/ml) for 20 h at 37°C and a standard alkaline-lysis protocol was followed (Silhavy et al. 1984). Inserts were excised with *Not*I and resolved on a CHEF gel.

To prepare high-density filters, Hybond N+ filters (115 × 75 mm) (Amersham Life Sciences, Arlington Heights, Ill., USA) were inoculated with the bacterial clones of four 384-well plates (a total of 1536 clones/filter) using the Nunc replication system. The inoculated filters were placed on Nunc OmniTrays containing LB agar with 12.5 μg/ml of CM and incubated at 37°C overnight until the colonies were 1.0–1.5 mm in diameter. The library of 30 720 BAC clones was blotted onto 20 high-density filters with two replications. Processing of the filters was performed as described (Woo et al. 1994). Hybridizations were carried out as reported previously (Danesh et al. 1994).

To prepare pools of BAC DNA for polymerase chain reaction (PCR) amplification screening (Green and Olson 1990), the BAC clones from four 384-well plates (1536 BAC clones) were replicated onto a Nunc OmniTray containing LB agar with 12.5 μg/ml of CM using the Nunc replication system. Sub-pools consisted of the 384 BAC clones from a single plate replicated onto an OmniTray as above. In either case, trays were incubated at 37°C until the colonies had grown to 1.5–2.0 mm in diameter. All the colonies in one tray were suspended in 20 ml of sterile water and then transferred into a 50-ml sterile tube. The cells were concentrated by centrifugation at 2 200 g for 5 min and the pellet re-suspended in 4 ml of GTE (50 mM glucose; 25 mM Tris-HCl, pH. 8.0; 10 mM EDTA) and a scaled-up alkaline-lysis protocol (Silhavy et al. 1984) was followed. The final DNA pellet was re-suspended in 0.5 ml of TE. A total of 20 DNA pools and 80 sub-pools were prepared for the library, with DNA concentrations ranging from 10 to 130 μg/ml. These pools and sub-pools were then screened by PCR amplification with specific oligonucleotide primers and gel-electrophoresis as described (Green and Olson 1990). To identify the BAC clone amplified by the primers, new pools from all of the wells in single columns and rows on the microtiter plate identified in the proceeding steps were prepared, followed by PCR-amplification and electrophoresis.

For dot-blot analysis, 6–12 ng of BAC DNA in 6 μl was denatured with 2 N NaOH. Two microliters were spotted onto Hybond N+ membranes and fixed by placing the membrane, DNA side up, on Whatman 3MM paper (Whatman International, Ltd., Maidstone, UK) soaked in 0.4 N NaOH for 20 min. The membrane was neutralized by placing it on Whatman 3MM paper soaked in 2 × SSC/0.2 M Tris-HCl (pH 7.5) for 10 min.

RFLP Analysis

To distinguish between clones hybridizing to duplicate RFLP loci, BACs were digested with the restriction enzyme originally used to map the RFLP, then probed with a radiolabelled copy of the RFLP clone. If the size of the hybridizing fragment derived from the BAC clone was identical to the genomic fragment hybridizing to the RFLP, we inferred that the BAC originated from the genomic segment corresponding to the RFLP. This was accomplished by digesting soybean genomic DNA from genotypes 'Evans', Faribault and PI 209332, as well as purified BAC DNA, with either *Dra*I, *Eco*RV, *Taq*I, or *Not*I, followed by blotting and hybridization as described (Young et al. 1992).

BAC-insert end isolation

To isolate end fragments of BAC inserts, BACs were first digested with *Hin*dIII in TA buffer. After heat-inactivation, the digested DNA was self-ligated at 16°C for 2–3 h. One to two microliters of the ligation reaction were then used in a 50-μl PCR reaction.

Primers AB3 and AB4 (kindly provided by A. Bendahmane and D. Baulcombe, Sainsbury Laboratory, UK) were employed for right-end isolations. T7 and BAC4 (Woo et al. 1994) oligonucleotide primers were used for amplification of the left-end. PCR reaction conditions were the same as reported in Cai et al. (1995).

Results and discussion

BAC library construction

A BAC library from the DNA of soybean cultivar Faribault was constructed using the *Eco*RI site of pECSBAC4 (Frijters et al. 1997). This enzyme is a reliable endonuclease that can be used in combination with *Eco*RI methylase. It also makes the BAC system compatible with *RecA*-assisted restriction endonuclease cleavage (Ferrin and Camerini-Otero 1994) and amplified fragment length polymorphism markers (Vos et al. 1995). Four separate ligation experiments yielded a BAC library consisting of 30 720 clones. To characterize the library, 50 BACs were digested with *Not*I (which excises the insert) and resolved by CHEF gel-electrophoresis. Even though we cloned genomic fragments migrating between 225 and 450 kbp (based on molecular-weight-markers run on the same gel), BAC inserts ranged in size from 40 and 230 kbp with an average of 120 kbp (Fig. 1). Presumably the larger fragments in the samples were not efficiently cloned, so that only the relatively smaller genomic fragments were recovered in the final library. Attempts to increase the average insert size through a second round of size-selection were unsuccessful. The inserts exhibited an approximately normal size distribution (Fig. 2). Based on an estimated genome size of 1.115 million kbp for soybean (Arumuganathan and Earle 1991) this library provides slightly greater than a threefold redundant genome representation with an approximately 95% chance of containing a BAC clone corresponding to any soybean DNA sequence.

To determine the extent of repetitive DNA sequences in the library, we prepared dot-blots of 40 BAC clones and probed the blots with radiolabelled soybean genomic DNA. The presence of high-, medium-, or low-copy sequences was then inferred from the intensities of individual BAC clones on the dot-blot. These results indicated that 55% of the clones contained high-copy sequences (dark to very dark signals), 25% contained medium-copy sequences (light signals), and 20% contained only low-copy sequences (faint or non-detectable signals).

Importance of the vector de-phosphorylation step

In the course of constructing the BAC library, we found that the vector de-phosphorylation step, which is essential in creating the library, was prone to difficulties. As in the construction of some other BAC libraries, we de-phosphorylated our plasmid DNA using HK phosphatase (Woo et al. 1994). Preliminary ligation experiments with de-phosphorylated vector stored at −80°C for 2 days produced satisfactory numbers of recombinant clones (Table 1). However, subsequent ligation reactions and transformations showed a gradual decline in the number of recombinant BAC clones, until only a few or no recombinant BACs could be generated (Table 1). Phosphorylation of 5-week-old de-phosphorylated vector DNA (stored at −80° C) using polynucleotide kinase followed by self-ligation with T4 ligase failed to generate distinct intact plasmid bands on an agarose gel (data not shown). However, ligation of size-selected, partially *Eco*RI digested soybean DNA into freshly de-phosphorylated plasmid vector followed by transformation led to a high proportion of

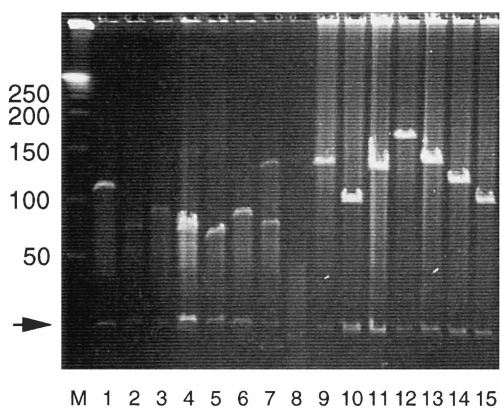

Fig. 1 CHEF gel electrophoresis of 15 BAC clones. DNA isolation, *Not*I digestion, and gel-electrophoresis as described in Materials and methods. *M*, molecular markers. *Arrow* points to the pECSBAC4 vector. The sample in *lane 7* is presumed to have an internal *Not*I site. The sample in *lane 8* is apparently degraded. Sizes are given in kbp

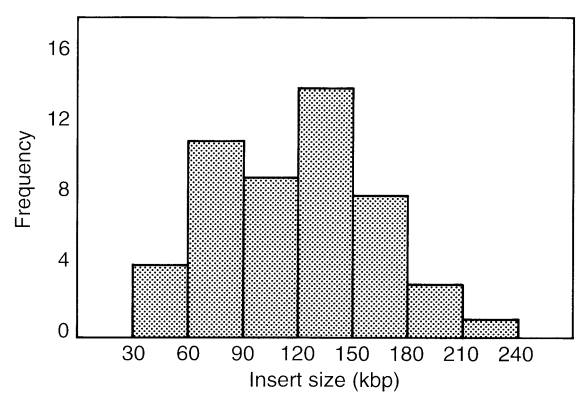

Fig. 2 Histogram showing the size distribution of 50 BAC-clone soybean genomic inserts

recombinant BAC clones. We concluded that de-phosphorylation led to a relatively rapid degradation of the plasmid DNA during storage.

Identification of BAC clones near a major SCN resistance gene

To screen the library with RFLPs, bacterial clones of four 384-well plates (for a total of 1536 clones) were transferred onto nylon membranes. The entire library could therefore be blotted onto 20 high-density filters. Four soybean RFLP clones (C006V, B053T, D0140, and Bng122D) that map near the major cyst nematode resistance gene on MLG-G of soybean (Danesh et al. 1995; Concibido et al. 1996) were used to screen the high-density filters. Recent experiments indicate that B053T is very tightly linked to this gene at approximately 1.7 cM on the telomeric side (Concibido et al. 1996; Young et al., unpublished observations).

For each of these RFLPs, at least one corresponding BAC clone was identified. In the case of B053T and C006V, three corresponding BACs were identified for each marker (Table 2). Soybean is thought to be a diploidized tetraploid, so it was possible that BAC clones found using an RFLP clone actually map to different, but homologous, loci (Shoemaker et al. 1996). To determine whether the BAC clones identified corresponded to the RFLPs on MLG-G, BAC clones were digested with the restriction enzyme originally used to map the RFLPs onto MLG-G and compared with genomic DNA digested with the same restriction enzyme. If the digested BAC and genomic DNAs both contained a restriction fragment of the same size after hybridization with the corresponding RFLP clone, we inferred that the BAC originated from the RFLP locus on MLG-G (Fig. 3). This process demonstrated that five of the BACs (one each for C006V, Bng122D and D0140, and two for B053T) were derived from sequences located on MLG-G. One of the clones identified with C006V and

Table 1 Effects of storage of de-phosphorylated vector on the transformation efficiencies of BAC clones[a]

Length of storage (days)	Number of white colonies/total	Percent false positives[b]
2	283/431	5
32	105/258	15
46	23/86	53
75	13/122	77
90[c]	30/239	100
1[c]	411/698	9

[a] Based on transformation using 1 µl of ligation reaction mixture and electroporation into *E. coli* DH10B cells as described in the Materials and methods
[b] White colonies (as described in the Materials and methods) subsequently determined to have no genomic insert
[c] Ligations carried out with same insert DNA, followed by parallel transformation and plating

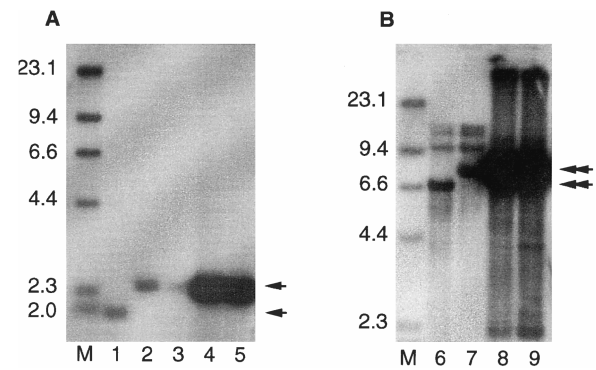

Fig. 3 Comparison between the restriction digest patterns of BAC clones and soybean genomic DNA probed with various RFLPs. **A** BAC-I18 hybridized with Bng122D; **B** BAC-J12 hybridized with C006V. *Lane M*, molecular-weight standards; *lanes 1 and 6*, Evans; *lanes 2 and 7*, PI 209332; *lane 3*, Faribault; *lanes 4 and 5*, BAC-I18; *lanes 8 and 9*, BAC-J12. *Lanes 1–4* digested with *Dra*I; *lane 5* digested with *Not*I and *Dra*I; *lanes 6–8* digested with *Eco*RV; *lane 9* digested with *Not*I and *Eco*RV. *Single arrows* denote the RFLP fragments used to map Bng122D; *double arrows* denote RFLP fragments used to map C006V. (Faribault is derived from PI 209332 and both have identical RFLP genotypes at Bng122D and at C006V). Sizes are given in kbp

Table 2 BAC clones identified with DNA markers located near the major SCN resistance gene on MLG-G

BAC clone nmae	DNA marker	Insert size (kbp)	Copy number	End-clone isolation	MLG-G position confirmed
K15	B053T	80	High	N.D.[a]	No
N16	B053T	90	Low	R, L[b]	Yes
M13	B053T	140	Med	R, L	Yes
I18	Bng122D	110	Med	Failed	Yes
K4	Satt309	75	N.D.	R	Yes
K9	D0140	100	N.D.	R, L	Yes
J12	C006V	120	Low	L	Yes
L13	C006V	100	N.D.	N.D.	N.D.
D2	C006V	230	N.D.	N.D.	No

[a] N.D., Not determined
[b] R, right end of BAC clone; L, left end of BAC clone

another identified with B053T appeared to map to different genomic locations (Table 2).

The library was also screened by the polymerase chain reaction (PCR) using pools of BAC clones as templates. A total of 20 DNA pools, four sub-pools, and 40 pools derived from rows and columns were screened with the microsatellite marker BARC-Satt309 (P. Cregan, USDA, Beltsville, Md., USA, personal communication). This marker maps only 0.4 cM away from the SCN resistance gene (Young et al., unpublished observations). A single BAC pool amplified a product of the correct size (data not shown). From this pool, a single BAC clone (K4) was eventually identified through PCR analysis of the corresponding sub-pools and pools derived from individual rows and columns (Table 2).

BAC-end isolation

For genomic analysis and chromosome walking, it is essential to recover the ends of BAC clone inserts. Typically, this is accomplished by plasmid rescue, PCR or inverse PCR (Woo et al. 1994). We have focused on the use of PCR and IPCR amplification to isolate the ends of BAC clone inserts. Specifically, we modified the method of Woo et al. (1994) to isolate ends without plasmid rescue. With this modification, as much as 5.0 ng of BAC DNA could be subjected to enzymatic digestion and processing in a single reaction tube without the need for DNA precipitation, reducing both time and the loss of DNA. This same strategy could be potentially used with alternate restriction enzymes surrounding the *Eco*RI site of pECSBAC4, such as *Sph*I.

Using this strategy, we isolated 8 of 12 ends from six of the BAC clones described above (Table 2). This approach to end-cloning failed to amplify the left end of BAC-K4, the right end of BAC-J12, and both ends of BAC-I18. One possible explanation for these failures could be that the restriction fragments generated by *Hin*dIII-digestion were too large for efficient PCR amplification. Alternative methods for cloning these ends are now underway.

In previous publications about the construction of BAC libraries in plants, researchers used in situ hybridization with BAC clones to monitor the stability of clones and (lack of) chimeras within the library (Woo et al. 1994). In these experiments, the presence of a single fluorescent locus under the microscope was used to indicate that the BAC clone was not a chimera. Unfortunately, this approach is unsuitable for soybean with its large amount of repetitive DNA and the existence of multiple homologous loci within the genome. Therefore, we carried out a combination of physical and genetic mapping using end- and internal-subclones derived from four of the BAC clones listed in Table 2 (M13, N16, K4, and K9). In each case, we found that end-clones (from M13 and N16) or subclones (from K4 and K9) co-segregated exactly or else mapped less than 1 cM away from the corresponding RFLP, indicating that the BACs were not chimeric. Though the number of clones tested so far is low, the results agree with previous observations that the level of chimeras is minimal in BAC libraries (Woo et al. 1994; Wang et al. 1995). These results also indicate that the recombination rate is relatively high in this region of the soybean genome, since crossovers between end- and sub-clones on individual BACs could be identified in two mapping populations of 218 individuals (Concibido et al. 1996; Young et al., unpublished observations).

Prospects for positional cloning

Success with positional gene cloning in plants demonstrates that this strategy can be effective with disease-resistance genes. Generally, progress has been greatest in plant species with small genomes (Bent et al. 1994; Grant et al. 1995; Song et al. 1995) or extensive classical and molecular-genetic resources (Martin et al. 1993). Recently, a gene was cloned based on map position in barley, a crop with a relatively large and complex genome (Büschges et al. 1997). There are still, however, no examples of successful positional gene cloning in soybean. The development of a threefold-redundant BAC library for soybean reported here, indexed and arrayed in 384-well microtiter plates, should facilitate positional cloning in this important crop species. Comparable BAC libraries for soybean have been developed in other laboratories (Marek and Shoemaker 1997; M. Bhattacharya, Ardmore, Okla., USA, personal communication). The library described in Marek and Shoemaker (1997), for example, was developed with partial *Hin*dIII fragments and is estimated to contain 4–5 genome equivalents, while the BAC library we describe here was constructed with partial *Eco*RI fragments. Because these libraries were constructed with different restriction enzymes, they complement one another and together represent a 7- to-8-fold redundant representation of the soybean genome, adequate for nearly any positional gene-cloning effort.

Positional cloning of plant disease-resistance genes, in particular, should proceed rapidly using tools such as our BAC library. Since resistance genes tend to be clustered, a BAC contig that spans one resistance gene may also span others (Martin et al. 1994). For example, there are now several examples of clustered resistance-gene analogs in soybean (Kanazin et al. 1996; Yu et al. 1996). Coupled with the approach of using conserved, but degenerate, PCR primers to search for putative resistance genes (Leister et al. 1996), BAC libraries provide a valuable tool for candidate gene isolation.

In the case of the major SCN resistance on MLG-G, we have already isolated BAC clones on three discontinuous contigs that span 365 kbp very near the target gene. Separately we have estimated the size of this

interval to be approximately 3.9 centimorgans in length (Concibido et al. 1996; Young et al., unpublished observations). Previously, the physical-to-genetic distance ratio in this region of the soybean genome was estimated at 150 kbp/centimorgan (Danesh et al. 1995) and results with the BACs reported here corroborate this estimate. Consequently, a significant proportion of the soybean genome where the SCN resistance gene resides has already been cloned. Currently, we are working to fill the physical gap between BAC clones, increase the size of the mapping population, and uncover additional DNA markers located near the gene.

Requests to screen the BAC library

Researchers who wish to screen this BAC library for clones of interest should contact Dr. Nevin Dale Young at e-mail: neviny@tc.umn.edu or Fax: 612-625-9728.

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