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A bacterial artificial chromosome library for soybean PI 437654 and identification of clones associated with cyst nematode resistance

J.P. Tomkins[1,*], R. Mahalingam[2], H. Smith[1], J.L. Goicoechea[1], H.T. Knap[3,*] and R.A. Wing[1,*]
[1]*Clemson University Genomics Institute, Room 100, Jordan Hall, Clemson, SC 29634, USA;* [2]*Biotechnology Institute, Pennsylvania State University, College Park, PA 16802, USA;* [3]*Dept. of Crop and Soil Environmental Sciences, Clemson University, Clemson, SC 29634, USA (*[*]*authors for correspondence)*

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Abstract

We have constructed a soybean bacterial artificial chromosome (BAC) library using the plant introduction (PI) 437654. The library contains 73 728 clones stored in 192 384-well microtiter plates. A random sampling of 230 BACs indicated an average insert size of 136 kb with a range of 20 to 325 kb, and less than 4% of the clones do not contain inserts. Ninety percent of BAC clones in the library have an average insert size greater than 100 kb. Based on a genome size of 1115 Mb, library coverage is 9 haploid genome equivalents. Screening the BAC library colony filters with cpDNA sequences showed that contamination of the genomic library with chloroplast clones was low (1.85%). Library screening with three genomic RFLP probes linked to soybean cyst nematode (SCN) resistance genes resulted in an average of 18 hits per probe (range 7 to 30). Two separate pools of forward and reverse suppression subtractive cDNAs obtained from SCN-infected and uninfected roots of PI 437654 were hybridized to the BAC library filters. The 488 BACs identified from positive signals were fingerprinted and analyzed using FPC software (version 4.0) resulting in 85 different contigs. Contigs were grouped and analyzed in three categories: (1) contigs of BAC clones which hybridized to forward subtracted cDNAs, (2) contigs of BAC clones which hybridized to reverse subtracted cDNAs, and (3) contigs of BAC clones which hybridized to both forward and reverse subtracted cDNAs. This protocol provides an estimate of the number of genomic regions involved in early resistance response to a pathogenic attack.

Introduction

The soybean cyst nematode (SCN), *Heterodera glycines* Ichninohe, is one of the most significant pests of modern cultivated soybean. The presence of SCN in the USA was first reported in North Carolina (Winstead *et al.*, 1955) and has spread to all of the major soybean producing states in the USA. Damage to SCN infection has been reported at more than USD143 million (634 000 tonnes, metric tons) per year in the southern USA where the pest is especially prominent (Sciumbato, 1993). Furthermore, the importance of using genetic resistance to SCN has increased substantially due to the ban on the nematicide methyl bromide (Noling and Becker, 1994) and the cancellation of permits for the use of DBCP (1,2-dibromo-3-chloropropane) and EDB (ethylene dibromide).

The presence of multiple physiological races in SCN (Riggs and Schmidt, 1988) has led to the development of cultivars with multiple SCN race resistance through traditional breeding techniques. The soybean germplasm pool in North America, however, has a limited base for SCN resistance genes. In a report by Lohnes and Bernard (1991), SCN resistance in 69/130 resistant cultivars was traced to only two ancestral genotypes ('Peking' and 'PI 88788').

Studies evaluating the segregation of SCN resistance have shown polygenic inheritance patterns (Mansur *et al.*, 1993). Further, the molecular mapping of genes associated with SCN has made significant progress using a number of SCN resistance

sources (Weisman *et al.*, 1992; Concibido *et al.*, 1994, 1997a,b; Webb *et al.*, 1995; Vierling *et al.*, 1996; Mahalingam and Skorupska, 1996; Chang *et al.*, 1997; Heer *et al.*, 1998). These maps provide the necessary guide for the physical mapping and cloning of multiple SCN resistance genes.

Diers *et al.* (1997) studied the genetic relationships among 38 primitive plant introductions containing resistance to one or more races of SCN and found two genotypes having resistance to all known SCN races: PI 438489B and PI 437654. However, unpublished data on PI 438489B indicate that it may not have complete resistance to race 14 (Rao-Arelli, personal communication). Therefore, PI 437654 stands alone as a germplasm source containing a complete complement of genes conferring broad resistance to SCN. By using PI 437654 as a DNA source for a large-insert genomic library, the genes conferring complete resistance to SCN in their primitive unrecombined form could be accessed for map-based cloning.

The map-based cloning of SCN resistance genes requires the development of large-insert genomic libraries. The bacterial artificial chromosome (BAC) cloning system appears to offer advantages over other large-insert cloning systems as discussed previously (Shizuya *et al.*, 1992; Woo *et al.*, 1994). Therefore, the BAC system is very appealing as a vehicle for advanced genome analysis in soybean. At the present time, a public soybean BAC library containing resistance genes to SCN race 3 has been developed from the northern USA cultivar Faribault (Danesh *et al.*, 1998). Another public soybean BAC library developed from cv. Williams 82 is also available, but does not contain SCN resistance genes (Marek and Shoemaker, 1997). At the present time, there are no reports describing soybean BAC libraries that would provide a broad spectrum of resistance genes to all known races of SCN.

In the current study, we report the development of a BAC library for soybean PI 437654, a primitive germplasm that contains resistance genes to all known races of SCN. The library was characterized with chloroplast DNA sequences and low-copy-number genomic clones linked to SCN resistance genes. We have also developed two cDNA pools from PI 437654 in response to early SCN infection using a suppression subtractive hybridization approach that enriches target cDNAs which correspond to rare transcripts. These cDNAs were then used to screen the BAC library to quantify and characterize genomic regions involved in early SCN resistance responses.

Materials and methods

BAC library construction

The single-copy BAC library vector pBeloBAC11 was obtained from Dr Hiroaki Shizuya (Shizuya *et al.*, 1992) and prepared as described by Woo *et al.* (1994). Megabase plant DNA embedded in agarose plugs was obtained as described by Zhang *et al.* (1996). Partial digests of megabase DNA were performed as described by Tomkins *et al.* (in press) with the following modifications: chopped plugs were distributed in 100 μl aliquots and incubated on ice for 30 min with 14 μl 10$\times$ enzyme buffer, 14 μl 40 mM spermidine, and 1.4 μl BSA. After a second 30 min incubation with 2 units *Hin*dIII on ice, digestion reactions were allowed to proceed at 37 °C for 30 min. Digestions were stopped by placing on ice and adding 1/10 vol. 5 M EDTA. Partially digested megabase DNA was subjected to two size selections by pulsed-field electrophoresis (CHEF mapper apparatus, BioRad). Initial size selection conditions were: 1% low-gelling-temperature agarose, 1–60 s linear ramp, 6 V/cm, 12 °C, 22 h run time, and 0.5$\times$ TBE buffer. Two fractions between 150 and 300 kb were cut from the gel based on a 50 kb lambda ladder reference (NEB). Gel slices were transferred to a second CHEF of similar composition and run at a constant 4 s switch time under similar time and temperature conditions. Two gel slices were excised and DNA was removed from the agarose by electroelution using the BioRad Electro-Eluter (Model 422) system. Ligations were performed in 150 μl reactions using 30 ng vector and 250 ng DNA and allowed to proceed for 20 h at 16 °C. After desalting ligations, transformations were performed using 2 μl ligation reaction and 20 μl competent cells (DH10B, Gibco/BRL). Electroporations were performed on a cell porator with voltage booster (Gibco/BRL) using 320 V at a resistance of 4 kΩ. Transformed cells were diluted immediately with 0.5 ml SOC (Sambrook *et al.*, 1989) and incubated at 37 °C for 60 min before being plated on selective medium (LB, Luria-Bertani medium) with 12.5 μg/μl chloramphenicol, 0.55 mM IPTG, and 80 μg/ml X-Gal. After a 20 h incubation at 37 °C, the plates were placed at room temperature in the dark for an additional 20 h to allow stronger color development of nonrecombinant colonies. After determining insert sizes of clones, a ligation derived from the 225 to 300 kb gel fraction was utilized for additional transformations to construct the library. Recombinant white

colonies were picked robotically (Genetix Q-bot) and stored individually in 192 384-well microtiter plates (Genetix) containing 50 μl freezing broth (Woo *et al.*, 1994). After incubation overnight, microtiter plates were stored at -80 °C. Two copies of the library were made and stored in separate -80 °C freezers.

BAC clone characterization

To prepare BAC DNA, 3 ml LB chloramphenicol (12.5 μg/μl) cultures were grown overnight in 6-cell autogen tubes and miniprepped robotically (Autogen 740 plasmid isolation system). To estimate insert size and determine distribution of clone size, a total of 230 BAC preps were performed from clones selected at random throughout the library. The BAC DNA was digested with 7.5 units (10 h at 37 °C) of *Not*I and analyzed by pulsed-field electrophoresis in 1% agarose gels (6 V/cm, 5–15 s switch time, 15 h run time, 14 °C). Southern blots of size-separated BAC inserts were performed using standard protocols (Sambrook *et al.*, 1989) after UV nicking the gels (Gene Linker, BioRad). Total genomic soybean DNA for use as probe was extracted from PI 437654 using the DNAzol ES extraction protocol for plants (Molecular Research Center) and ^{32}P-labeled using standard random priming techniques.

BAC library screening

High-density colony filters for hybridization-based screening of the library were prepared with the Genetix Q-bot. Clones were gridded in double spots using a 4 $\times$ 4 array on 22.5 cm^2 Hybond N+ filters (Amersham). This gridding pattern allows 18 436 clones to be represented per filter. Colony filters were treated and hybridized using standard techniques (Sambrook *et al.*, 1989). Radiolabeling [^{32}P] of probe DNA and hybridization of colony filters was performed using standard techniques (Sambrook *et al.*, 1989). Screening for chloroplast DNA in the library utilized three barley chloroplast clones containing *ndhA* (470 bp), *rbcL* (1300 bp), and *psbA* (1400 bp) sequences. These sequences are spaced equidistantly around the 133 kb barley chloroplast genome. Chloroplast clones were obtained from J. DuBell (Dept. of Biochemistry and Biophysics, Texas A&M University, College Station). Screening with three low-copy-number genomic clones linked to SCN resistance genes was also performed. Two soybean genomic clones, pK069 and pA110, and one *Phaseolus* genomic clone, BNG122, were used. Soybean clones were obtained from Biogenetic Services, and BNG122 was obtained from C.E. Vallejos (Horticultural Sciences Dept., University of Florida, Gainesville). Information on these clones and their genetic map locations in soybean can be viewed at the Soybase website (http://129.186.26.94).

BAC fingerprinting

To prepare BAC DNA for fingerprinting, 3 ml LB chloramphenicol (12.5 μg/μl) cultures were grown overnight in 5-cell autogen tubes and miniprepped robotically (Autogen 740 plasmid isolation system). BAC DNA was resuspended in 30 μl $TE_{10:1}$. Fingerprinting digestions were set up as follows: 4 μl BAC DNA, 1 μl *Hin*dIII buffer, 0.1 μl BSA, 0.5 μl *Hin*dIII (80 u/μl), and 4.4 μl sterile double-distilled H_20. Digestions were allowed to proceed at 37 °C for at least 4 h.

Gels were made using 150 ml of 1% Seakem LE agarose (FMC) in 1$\times$ TAE buffer. After cooling to 45 °C, molten agarose was poured into 20 cm $\times$ 25 cm UV transparent trays (Life Technologies) resting on a level surface giving a gel thickness of 3.5 mm. A 61-well comb (2 mm wide, 1 mm thick, 3 mm deep) was then inserted. After cooling, gels were loaded into precooled (16 °C) Model H4 electrophoresis units (Life Technologies). Temperature regulation was achieved by recirculating 1$\times$ TAE buffer through 7.5 m of plastic tubing immersed in a refrigerated water bath (VWR Scientific, model 1170). Only 4 μl of the BAC digestion reaction was used for loading the fingerprinting gel and the excess was stored at -20 °C. Standard marker DNA samples (1 μl) were loaded every fifth lane allowing 48 BAC fingerprint samples to be run per gel. Marker standards were a mixture of the following components in concentrations of 0.1 μg/μl each: Hi-Lo marker DNA (Minnesota Molecular) and three different stocks of lambda DNA cut with *Hin*dIII, *Stu*I, and *Sal*I. Gels were run at 90 V for 10 h. After electrophoresis, gels were stained for 1 h in SYBR Gold (Molecular Probes) and then imaged with a BioRad Fluor-S Multi Imager. Gel images were first cropped and then transferred as TIFF images to Unix workstations for band calling and contig building. Processing and digitizing of fingerprint gel images with Image software (Sanger Centre, UK) and building of contigs with FPC software (version 4.0, Sanger Centre) have been described in detail by Marra *et al.* (1997). Parameters used in our FPC analyses were as follows: tolerance value 7 and cutoff score 10^{-9}.

Development of cDNA pools

Seeds of PI 437654 were germinated as described by Mahalingam and Skorupska (1996). Inoculations with soybean cyst nematode (SCN) race 3 were performed with 3-day old seedlings as described in Mahalingam *et al.* (1998). Uninfected control plants were mock-inoculated with distilled water. About 2–3 cm root segments from the elongation zone were harvested 34 h after inoculation from infected and uninfected seedlings. Messenger RNA was isolated from the infected and control roots using the mRNA Fast Track Isolation Kit (Invitrogen). About 2 μg of mRNA from root tissue was used for the suppression subtractive hybridization procedure. For forward subtraction, mRNA from SCN-infected seedlings served as tester while mRNA from the uninfected plants formed the driver. For the reverse subtraction, mRNA from the uninfected seedlings served as tester and the driver was mRNA from the infected plants. cDNA synthesis, adaptor ligation, hybridizations and PCR amplifications were performed as described in the suppression subtractive hybridization kit (Clontech). Efficiency of subtraction was determined using soybean IOTA gene-specific primers. This gene is induced slightly after SCN infection (34 h). In the forward pool, this gene was enriched as determined by 10, 20 and 30 cycles of PCR. Using the reverse subtracted cDNA pool, no PCR product for the IOTA gene was obtained even after 30 PCR cycles indicating the subtraction was optimal. A detailed characterization of the cDNA pools is currently in progress and will be presented in a future publication.

For use as BAC colony filter probes, the secondary PCR amplification products from the forward and reverse subtractions were labeled with ^{32}P by random priming and labeled cDNAs were purified with NucTrap probe purification columns (Stratagene) and used as probes. The labeled cDNAs were then hybridized to the BAC library filters with the hybridization experiments being replicated in time and space.

Results

BAC library construction and characterization

We have constructed a BAC library for soybean PI 437654 which is suitable for physical mapping and cloning genes associated with SCN resistance. *Hin*dIII was used as the cloning enzyme because complete digests with soybean DNA produced fragments $\leq$ 30 kb. The library consists of 73 728 clones stored in 192 384-well microtiter plates. About 4% of the clones do not contain inserts as judged by random analysis of BACs sampled from the library. A random sampling of 230 BACs taken from the library indicated an average insert size of 136 kb with a range of 20 to 325 kb. Based on a haploid genome size of 1115 Mb (Arumaganthan and Earl, 1991) the coverage of the library is about 9 genome equivalents, resulting in a 99% probability of recovering any specific sequence. Figure 1A shows 28 randomly selected clones digested with *Not*I to release the insert. The two *Not*I sites in pBeloBAC11 flank the multicloning site. Because *Not*I is a GC-8-base cutter and the soybean genome is relatively AT-rich, digestion typically generates a vector band plus one insert band based on our data. Figure 1B shows a Southern blot of the gel in Figure 1A probed with total soybean genomic DNA indicating that the source of cloned DNA originated from soybean. As indicated by the strongly hybridizing lanes, many of the BACs contain highly repetitive DNA. However, 28% of the BACs contain primarily low-copy DNA as indicated by an insert band not hybridizing or weakly hybridizing on the southern blot.

To determine the size distribution of BAC clones in the library, the 230 BACs analyzed with *Not*I digests were grouped by insert size and the insert size of each clone was plotted against the frequency of each group of clones represented in the library (Figure 2). Based on this analysis, 90% of the clones in the library have an average insert size equal to or greater than 100 kb. Of the clones larger than 100 kb, 92% are equal to or greater than 120 kb.

To obtain an estimate of the representation of chloroplast DNA in the library, colony filters were screened with three different chloroplast genes. Results from this screening showed that ca. 1.85% of library sequences are chloroplast DNA (data not shown). The low chloroplast DNA content of the library was due to the use of nuclei as a megabase DNA source rather than protoplasts.

In order to evaluate the potential of using the library for map-based cloning, screening of colony filters was performed using three different low-copy-number genomic clones linked to SCN resistance loci on linkage groups A and G (Table 1). The clones BNG122 and pK069 identified 7 and 17 BAC clones, respectively while the pA110 clone identified 30 BACs. The results were generally indicative of the large genome coverage provided by the library based

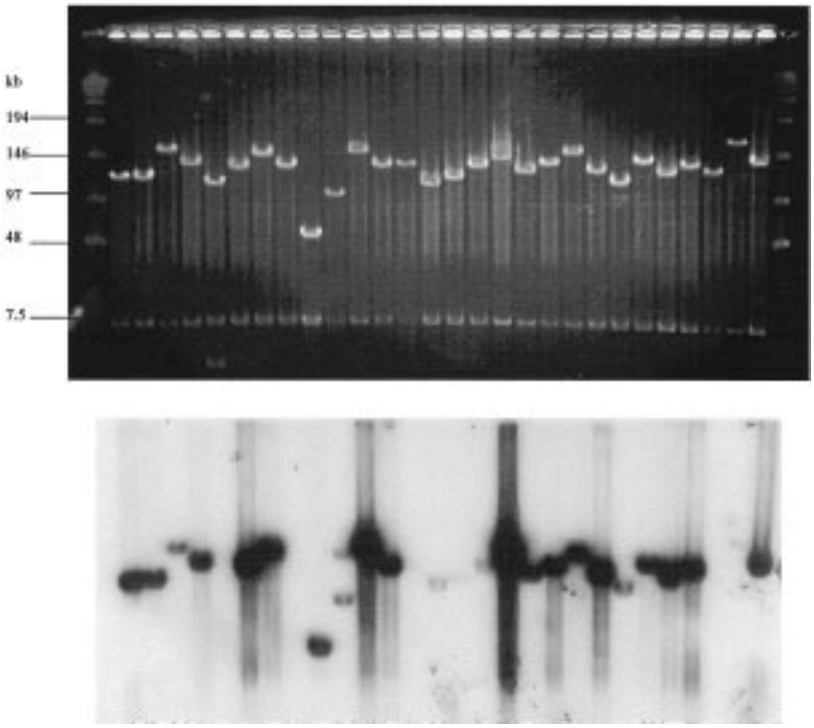

Figure 1. Analysis of 28 randomly selected soybean PI 437654 BAC clones. A (top). Ethidium bromide-stained CHEF gel (5–15 s switch time, 14 h) showing insert DNA above the common 7.5 kb pBeloBAC11 vector band. B (bottom). Autoradiograph of gel in A after Southern transfer and probing with total PI 437654 genomic DNA. Molecular weight marker in outside lanes is a 48.5 kb lambda concatamer (BioRad).

Table 1. Fingerprinting/contig analysis results of clones obtained from screening the soybean PI 437654 BAC library with three low-copy-number genomic clones linked to SCN resistance loci.

Probe[a]	Probe description	Clones identified	FPC contigs
pA110	Soybean genomic clone	30	7
pK069	Soybean genomic clone	17	3
BNG122	*Phaseolus* genomic clone	7	2

[a]pK069 and BNG122 are linked to a SCN resistance locus on linkage group G and pA110 is linked to a SCN resistance locus on linkage group A.

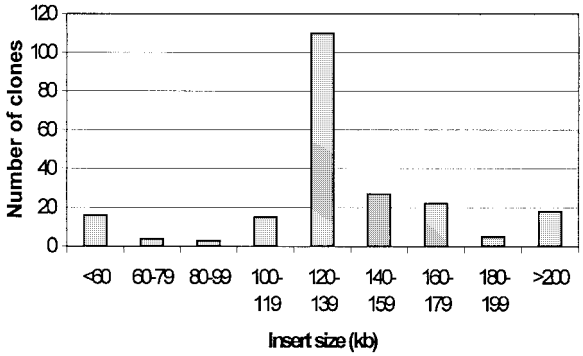

Figure 2. Insert size distribution of BAC clones in the soybean PI 437654 BAC library. To estimate insert size range, BAC DNA from 230 randomly selected clones were analyzed, as shown in Figure 1A. Results indicate that the average insert size is 136 kb with over 90% of the clones >100 kb.

on an average of 18 hits per probing. Results were probably also indicative of the duplicated nature of the soybean genome which is thought to be an ancient polyploid (Hadley and Hymowitz, 1973; Shoemaker *et al.*, 1996). In order to determine relationships between clones identified with various probes, clones were *Hin*dIII-fingerprinted, gel images scanned into a computer, digitized using IMAGE software (Sanger Centre, UK) and analyzed using FPC (Sanger Centre). Fingerprinting of BAC clones and analysis with FPC has previously been described in detail by Marra *et al.* (1997). Results from FPC analysis showed that contigs could be easily formed with related soybean BAC clones. The number of contigs formed for each set of BAC clones identified with a particular probe are shown in Table 1.

Screening the BAC library with cDNA pools

Two separate pools of cDNAs were developed as described in Materials and methods. The cDNAs represent selectively amplified differentially expressed genes involved in early response to SCN infection in root tissue. Using enriched cDNA subtraction techniques, target cDNAs that correspond to rare transcripts were obtained in quantities sufficient for use as probes for hybridization-based experiments. The first pool (forward subtraction) represented transcribed genes associated with early response to SCN infection (34 h after inoculation). The second pool (reverse subtraction) represented genes which were down-regulated in response to SCN infection. Each pool was ^{32}P-labeled and hybridized separately to the PI 437654 BAC library in replicate. After identifying the library addresses of each positive signal, BACs were fingerprinted and subjected to FPC analysis.

BAC colony filter hybridization with the forward and reverse subtraction cDNAs resulted in a total of 404 and 145 positive signals, respectively. Fingerprint and FPC contig analyses of BAC clones corresponding to the hybridization signals resulted in contigs which were placed in one of three categories: (1) contigs of BAC clones which hybridized to forward subtracted cDNAs, (2) contigs of BAC clones which hybridized to reverse subtracted cDNAs, and (3) contigs of BAC clones which hybridized to both forward and reverse subtracted cDNAs. The number of contigs formed in categories 1, 2, and 3 were 52, 4, and 29 respectively. Each FPC contig appears to represent a unique genomic region (Tomkins *et al.*, in press). Based on these data, 52 genomic regions are associated with increased transcriptional activity in response to SCN infection while only 4 regions are down-regulated. It is difficult to interpret the nature of transcriptional activity in the 29 genomic regions that are associated with both an increase and decrease in transcriptional activity. Because the BAC clones contain large stretches of DNA which may contain a number of genes or gene clusters, it is possible that these regions might contain both up-regulated and down-regulated sequences.

To illustrate the size distribution of contigs in each category, the contigs were placed in ascending order based on contig size (number of clones in the contig) in three histograms corresponding to each contig category (Figure 3). For the first contig category (up-regulated genomic regions), 85% of the contigs contained only 2 to 4 clones while 15% of the contigs contained 6 to 18 clones and the average was 4 clones per contig. For the second category (down-regulated genomic regions), contig sizes ranged from 3 to 16 clones with an average of 8 clones per contig. For the third category (regions showing both up- and down-regulation), contig sizes ranged from 2 to 24 clones with an average of 7 clones per contig. Interestingly, contigs in the second and third categories did not form groups of contigs of the same size in the histogram as did the contigs in the first category. Also, the average contig size in the second and third categories was about twice as large as average contig size in the first category. Of further note, the total number of BAC clones were nearly similar (203 vs. 206) between the first and third categories although the number of contigs was nearly reduced by half in the third category.

Discussion

In the present paper, we describe the development and characterization of a high-quality BAC library for soybean using the ancestral germplasm PI 437654. This large-insert library provides an important resource to perform the map-based cloning of all known genes conferring resistance to SCN, one of the most significant pests of modern cultivated soybean. The library will also furnish a genetic resource for genes present in primitive germplasm. Because the library provides nine haploid genome equivalents, the likelihood of finding any sequence of interest is greatly increased. The library has been deposited in the Clemson University Genomics Institute BAC library resource center and is publicly available. Requests for colony filters and clones can be made by accessing the Clemson University Genomics Institute web page (www.genome.clemson.edu).

The utility of the library for map-based cloning experiments was demonstrated by screening the BAC colony filters with three low-copy-number genomic clones linked to SCN resistance genes on two different linkage groups. The results were indicative of the large genome coverage provided by the library as an average of 18 hits per probing were obtained for the three genomic clones. However, an increased number of hits for the pA110 clone suggested that some regions of the genome may be preferentially cloned. It is likely that the large number of hits per probing also demonstrated that soybean is an ancient polyploid. *Hin*dIII fingerprinting and contig analysis of the corresponding BAC clones provided an efficient method of placing related

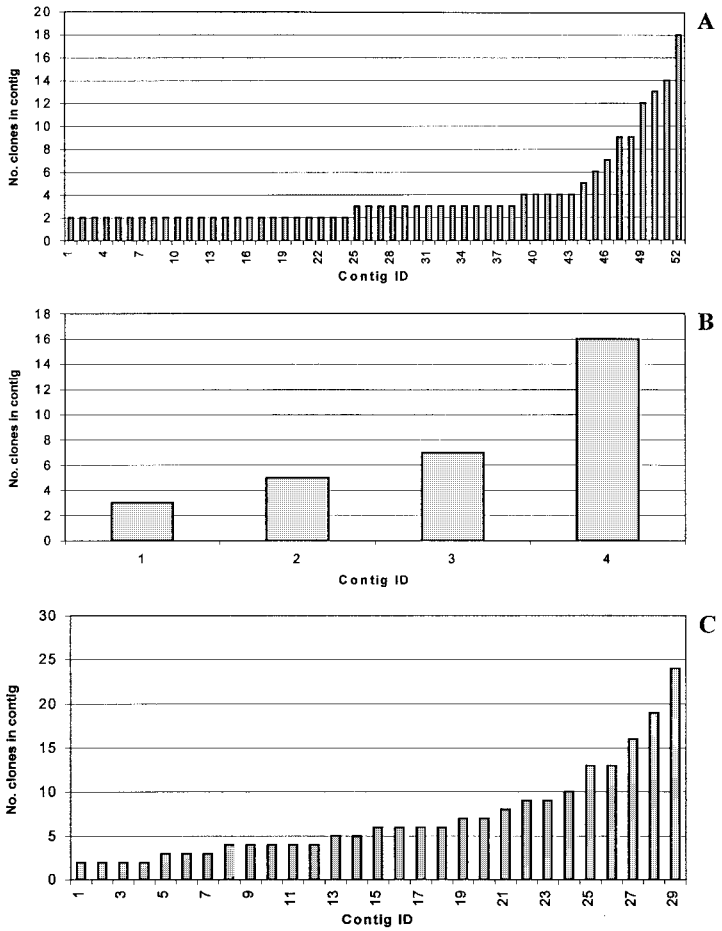

Figure 3. Graphs showing the distribution of number of BAC clones per contig in three hybridization categories. Fingerprinted BACs were those identified from positive signals after hybridizing differentially expressed cDNA pools to the BAC library. A. Contigs from BACs which hybridized only to cDNAs derived from up-regulated genes in response to SCN infection. B. Contigs from BACs which hybridized only to cDNAs derived from down-regulated genes in response to SCN infection. C. Contigs from BACs which hybridized to both cDNA pools.

clones in specific groups. This protocol is necessary in a complex duplicated genome like that of soybean.

Using subtractive hybridization techniques, we have described the development of two cDNA pools which correspond to genes associated with early response to SCN infection of roots in PI 437654. The two separate cDNA pools represent genes which are up-regulated and down-regulated in response to SCN infection. Following the hybridization of the labeled cDNA pools to BAC colony filters, we were able to identify BAC clones directly associated with SCN resistance responses. However, it should be noted that many of the BAC clones may be associated with general host defense mechanisms.

In order to determine the number of genomic regions involved in early response to SCN infection, the BAC clones were *Hin*dIII fingerprinted and subjected to contig analysis. Results from this procedure showed the number of unique genomic regions involved in up-regulation, down-regulation, and also indicated regions involved in both up- and down-regulation. Presumably, each FPC BAC contig in an analysis represents a unique genomic region. While cloning and sequencing cDNAs can also provide a measure of the different types of genes being regulated in response to a stimulus, it is costly, more labor-intensive, and does not allow the complete testing of the entire cDNA pool as does the hybridization approach to a large insert BAC library with multiple genome equivalents. The approach we have presented here may enable the quantification of transcriptional responses in a relatively cost-effective, accurate, and timely manner. Data gathered from this type of experiment could then be used to estimate the number of

cDNAs which need to be sequenced. If desired, cDNAs of interest could then be hybridized back to the BAC library to obtain the complete genomic sequence including regulatory elements.

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