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Construction of a bacterial artificial chromosome (BAC) library for citrus and identification of BAC contigs containing resistance gene candidates

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Abstract A BAC library was constructed from the genomic DNA of an intergeneric *Citrus* and *Poncirus* hybrid. The library consists of 24,576 clones with an average insert size of 115 kb, representing approximately seven haploid genome equivalents and is able to give a greater than 99% probability of isolating single-copy citrus DNA sequences from this library. High-density colony hybridization-based library screening was performed using DNA markers linked to the citrus tristeza virus (CTV) resistance gene and citrus disease resistance gene candidate (RGC) sequences. Between four and eight clones were isolated with each of the CTV resistance gene-linked markers, which agrees with the library's predicted genome coverage. Three hundred and twenty-two clones were identified using 13 previously cloned citrus RGC sequences as probes in library screening. One to four fragments in each BAC were shown to hybridize with RGC sequences. One hundred and nine of the RGC BAC clones were fingerprinted using a sequencing gel-based procedure. From the fingerprints, 25 contigs were assembled, each having a size of 120–250 kb and consisting of 2–11 clones. These results indicate that the library is a useful resource for BAC contig construction and molecular isolation of disease resistance genes.

Keywords Citrus · BAC library · CTV resistance gene · Resistance gene candidates

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Introduction

Cultivated *Citrus* species are susceptible to a wide spectrum of pathogens including fungi, bacteria, nematodes, viruses, and viroids. The most serious viral pathogen is citrus tristeza virus (CTV), which induces two diseases: 'quick decline' or death of citrus trees on sour orange rootstock and 'stem-pitting' of scion cultivars independent of rootstock (Bar-Joseph et al. 1989). These diseases have caused huge losses and present a constant threat to citrus industries worldwide. Although some pummelo (*C. grandis* L. Osb.) accessions were found recently to be resistant to certain CTV strains (Garnsey et al. 1997), there is no genetic resistance to CTV in most *Citrus* species. *Poncirus trifoliata* L. Raf. (a close relative of *Citrus*), however, is resistant to a wide diversity of CTV strains (Garnsey et al. 1987; Yoshida 1985). Introgression of this resistance into rootstock cultivars has been successful via sexual hybridization, but the development of CTV-resistant and commercially acceptable scion cultivars has been very difficult due to the coincident introgression of undesirable fruit characteristics from *Poncirus*. Genetic studies indicated that the CTV resistance trait is controlled by a single dominant gene designated as *Ctv* (Fang et al. 1998; Gmitter et al. 1996). Molecular cloning of this gene would provide the means to develop resistant scion cultivars through genetic transformation. Toward this end, the *Ctv* gene region has been finely mapped, and DNA markers flanking and co-segregating with *Ctv* have been developed (Deng et al. unpublished; Fang et al. 1998).

The map-based cloning (MBC) strategy has been used successfully in the cloning of plant disease resistance (*R*) genes (Bent et al. 1994; Martin et al. 1993; Mindrinos et al. 1994). Cloning a target *R* gene using this strategy requires a fine genetic map and a number of closely linked DNA markers, as well as a large-insert genomic DNA library. In recent years, the bacterial artificial chromosome (BAC; Shizuya et al. 1992) system has been widely used for large-insert library construction. BACs are useful for cloning and maintaining large DNA frag-

ments, easy to recover and manipulate, and low in clone chimerism (Tao et al. 1994; Woo et al. 1994; Zhang et al. 1996a, b). To facilitate map-based cloning of *Ctv*, Gmitter et al. (1998) constructed a citrus BAC library containing 10560 clones with an average insert size of 80 kb, thus providing approximately 2× haploid genome coverage. Chromosome walking toward *Ctv* was initiated using this library, but walking progress was slow due to shallow genome coverage and small DNA inserts. A library of larger DNA inserts and a better genome coverage was, therefore, needed for efficient chromosome walking to the target gene (Zhang et al. 1996b; Zhang and Wing 1997).

The recent cloning of numerous plant *R* genes has led to the development of simple polymerase chain reaction (PCR)-based approaches to gain access to many *R* genes that were not readily accessible before (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). PCR amplification using degenerate primers designed from the conserved NBS (nucleotide-binding site) motif of several cloned plant *R* genes has resulted in the identification of many *R* gene-like sequences (Kanazin et al. 1996; Leister et al. 1996; Michelmore 1996; Yu et al. 1996). These sequences are called RGAs (resistance gene analogs; Kanazin et al. 1996), or RGCs (resistance gene candidates; Shen et al. 1998). Many of the RGCs are associated with known gene loci conferring resistance to viruses, bacteria, fungi, or nematodes (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996); some of them have led to the cloning of target resistance genes (Meyers et al. 1998). RGCs, therefore, seem to be useful in mapping and cloning plant *R* genes (Marek and Shoemaker 1997). Previous studies also showed that *R* genes and RGCs tend to cluster in plant genomes, and it is necessary to analyze large DNA fragments like BAC inserts or BAC contigs to understand the organization of *R* genes or RGC clusters (Marek and Shoemaker 1997).

Using PCR amplification of citrus genomic DNA with degenerate primers designed from the NBS motifs of Arabidopsis *RPS2* (Bent et al. 1994; Mindrinos et al. 1994), tobacco *N* (Whitham et al. 1994), and flax *L6* (Lawrence et al. 1995), Deng et al. (2000) identified 22 sequences similar to the NBS-LRR (leucine-rich repeat) class *R* genes. One RGC fragment is closely linked to *Ctv*; another one seems to co-segregate with *Ctv* and might lead to direct landing on the resistance gene (unpublished data). NBS RGC sequences are associated also with *Tyr1*, the major locus controlling citrus nematode resistance in *Poncirus* (Deng et al. 2000; Ling et al. 2000). Here we describe the construction and characterization of a large-insert BAC library from a CTV-resistant intergeneric hybrid of *Poncirus* and *Citrus* and the identification of BAC clones and BAC contigs containing *R*-gene candidates.

Materials and methods

BAC library construction

Tender leaves of USDA 17–47 [Thong Dee pummelo (*C. grandis*) × Pomeroy trifoliate orange (*P. trifoliata*)] were collected from mature trees, frozen in liquid nitrogen, and stored at –80°C. USDA 17–47 is the donor of the *Ctv* gene region that is under fine-resolution genetic mapping for positional cloning. Megabase DNA preparation, BAC cloning, and library construction were performed according to Zhang et al. (1995, 1996a, b). Nuclei of leaf cells were isolated and embedded in low-melting-point (LMP) agarose plugs (approximately 100 µl/plug). The nuclei were lysed, and megabase DNA was purified in the agarose plugs. Plugs containing purified megabase genomic DNA were stored in 50 m*M* EDTA solution at 4°C. Prior to partial digestion with *Bam*HI, the plugs were washed three times (1 h each time) by soaking in 10–20 vol of ice-cold TE buffer (10 m*M* TRIS-HCl pH 8.0, 1 m*M* EDTA pH 8.0) plus 0.1 m*M* phenylmethyl sulfonyl fluoride (PMSF), and three times in 10–20 vol of ice-cold TE buffer without PMSF on ice. Each plug was cut into nine smaller pieces approximately equal in size and these were incubated with 1× REact 3 buffer (Gibco BRL, Grand Island, N.Y.), and 2 m*M* spermidine, 1 m*M* DTT (dithiothreitol), and 0.2 mg/ml BSA (bovine serum albumin) on ice for 1 h. Various amounts of *Bam*HI were added to the plug pieces, and reactions were incubated on ice for an additional 1 h. After this, the reactions were transferred to a 37°C water bath, incubated for 5 min, and then stopped by adding 1/10 volume of 0.5 *M* EDTA pH 8.0. The partial digests were analyzed for fragment size distribution on a pulsed-field gel (1% agarose) using a CHEF DRIII apparatus (Bio-Rad, Richmond, Calif.). The electrophoresis was run at 6 V/cm, 11°C, a switch time of 50 s, and in 0.5× TBE buffer (45 m*M* Trizma base, 45 m*M* boric acid, and 1.0 m*M* EDTA, pH 8.3) for 18 h. The partial digestion conditions that yielded a majority of restricted DNA fragments ranging from 100 kb to 400 kb in size were selected and used for large-scale partial digestion.

Large-scale partial digests were size-selected on a 1% LMP agarose gel in 0.5× TBE buffer with the following electrophoresis conditions: 90-s switch time, 6 V/cm, 11°C, for 20 h. The gel region containing 100- to 400-kb DNA fragments was collected, and the DNA fragments were recovered from the agarose gel slices by electroelution. These DNA molecules were ligated with a *Bam*HI-digested and dephosphorylated pBeloBAC11 vector (Kim et al. 1996). The ligated DNA was used to transform *E. coli* ElectroMAX DH10B cells (Gibco BRL) by electroporation according to Zhang et al. (1996a). A Cell Porator and Voltage Booster System (Gibco BRL) was used; the Cell Porator was set at 350 V, 330 µF capacitance, low ohm impedance and fast charge rate, and the Voltage Booster was set at 4000 Ω. Transformed cells were cultured in SOC medium (Sambrook et al. 1989) at 37°C for 1 hour and then plated on LB agar medium (Sambrook et al. 1989) containing 12.5 µg/µl chloramphenicol, 0.55 m*M* IPTG, and 80 µg/ml X-Gal. White colonies were picked into 384-well microtiter plates containing 50 µl of freezing medium in each well (Zhang et al. 1996a). After incubation overnight at 37°C, the microtiter plates were frozen and stored at –80°C. From the original copy of the BAC library, two more copies were reproduced and stored in separate freezers.

BAC library screening

High-density colony filters were prepared using the Beckman BIOMEK 2000 Robotic Workstation. BAC clones were gridded in double spots using a 3×3 array, and 1536 clones were spotted onto each 8×12-cm Hybond-N+ membrane filter (Amersham-Pharmacia Biotech, Piscataway, N.J.). Colonies were grown on the filters at 37°C for 16 h before the filters were processed as described by Zhang et al. (1996a) and baked at 80°C for 2 h. For library screening, each set of 16 filters was pre-hybridized in a plastic box containing 135 ml of hybridization buffer (5× SSC, 5× Denhardt's so-

lution, 0.5% SDS, 25 m*M* potassium phosphate buffer, pH 6.5) at 65°C for 2–4 h. DNA probes were prepared from plasmid minipreps. Vector sequences were removed by digestion with appropriate restriction enzymes followed by agarose gel electrophoresis; probe DNA was recovered then from the gel and cleaned using GeneClean kit (Bio 101, Carlsbad, Calif.). The probe DNA was labeled with [^{32}P]-dCTP by random priming according to the manufacturer's recommendations (Amersham-Pharmacia Biotech) and added to the pre-hybridization buffer. Hybridization was carried out overnight at 65°C in an incubator with gentle shaking. Filters were washed at 65°C, twice with 0.5× SSC + 0.1% SDS for 20 min each wash, and once with 0.2×SSC + 0.1% SDS for 15 min. After washing, the filters were blotted dry, wrapped in plastic wrap, and exposed to Kodak X-OMAT AR films with a single intensifying screen at –80°C.

BAC characterization

BAC clones were inoculated in 5 ml LB broth (Sambrook et al. 1989) containing chloramphenicol (12.5 µg/ml) and grown overnight at 37°C with agitation at 250 rpm. Cells were harvested and DNA isolated using the alkaline lysis method as described by Zhang et al. (1996a). For insert size estimation, 7 µl of BAC DNA miniprep were incubated at 37°C for 3 h with 3 U of *Not*I enzyme in a 30-µl reaction containing 1× REact3 (Gibco BRL) and 2 mM spermidine. The *Not*I digests were separated by pulsed-field gel electrophoresis in a 1% agarose gel in 0.5× TBE buffer using a CHEF DRIII apparatus. The electrophoresis was run with an initial pulse time of 5 s, a final pulse time of 15 s, a voltage of 6 V/cm, and a temperature 11°C for 16 h. The gel was stained with ethidium bromide, destained in water for 30 min, and photographed. For Southern analysis, BAC DNA was digested with appropriate restriction enzymes and run on pulse-field gels as described above. The gels were soaked in 0.25 *N* HCl for 20 min, in water for 10 min, and then in 0.4 *N* NaOH for 20 min, before the BAC DNA fragments were transferred onto Hybond N+ nylon membrane filters under alkaline conditions (0.4 *N* NaOH). Filters were pre-hybridized, hybridized, and washed as described above for library screening.

BAC fingerprinting and contig assembling

BAC DNA was purified from a 5-ml aliquot of overnight culture using an improved alkaline lysis procedure and fingerprinted (Zhang and Tao, personal communication). Briefly, BAC DNA was double-digested, and the ends of the restricted fragment were labeled with [^{33}P]-dATP. The labeling reactions were stopped with the sequencing gel loading dye (98% v/v deionized formamide, 0.3% bromphenol blue, 0.3% xylene cyanol and 10 m*M* EDTA pH 8.0), denatured at 95°C for 5 min, and then subjected to electrophoresis on a 3.5% denaturing polyacrylamide gel in 1× TBE. Electrophoresis was performed on a SequiGen GT apparatus (Bio-Rad) for 100 min at 85 W. The gel was then transferred onto a sheet of 3MM blotting paper, dried under vacuum, and autoradiographed to X-ray film. The fingerprints on the autoradiographs were digitized using a UMAX scanner, and the image files were transferred to a SUN Ultra 10 workstation (SUN Microsystems, USA) for fingerprint editing and analysis. Fingerprint bands were identified and edited with the program IMAGE 3.9A (The Sanger Center, UK). Overlapping BAC clones were assembled into contigs using the program FPC (Soderlund et al. 1997) with a tolerance value of 2 and a cut-off value of 1e^{-20}.

Results

BAC library construction and characterization

USDA 17–47 was used as the genomic DNA source for BAC library construction because it is the pollen parent of a large BC1 population used in fine genetic mapping of the *Ctv* region. In addition, this accession carries both the CTV resistance and susceptibility alleles; therefore, a library derived from this source should enable the development of BAC contigs covering the two allelic regions and the isolation of the two alleles. To prepare megabase DNA suitable for BAC library construction, we evaluated several procedures. Embedding nuclei in LMP agarose plugs followed by cutting the plugs into small pieces consistently yielded higher amounts of DNA and much better reproducibility with partial digestion. Other evaluated procedures, including embedding protoplasts in agarose plugs or nuclei in microbeads, had problems with unstable partial digestions or low DNA yields.

Megabase DNA was partially digested with *Bam*HI, and the resulting DNA fragments were size-selected once on pulse-field gels. The recovered DNA was cloned into the *Bam*HI site of the pBeloBAC11 vector; BACs were maintained in *E. coli* strain DH10B. A total of 24576 clones were picked and stored in sixty-four 384-well microtiter plates. During the course of library construction and library screening process, we analyzed the insert sizes of more than 160 BAC clones. The average insert size of these sampled clones was 115 kb, with an insert size range of 60–230 kb (Fig. 1). The frequency of clones containing no inserts was less than 3%. The haploid genome size of citrus was estimated to be around 385 Mb (Arumuganathan and Earle, 1991). Based on this estimation and the library's average insert size, the coverage of this library is at least seven haploid genome equivalents, and the probability of recovering any sequences of citrus genomic DNA from this library is greater than 99%. Typically, one insert band per clone was observed after *Not*I digestion of BAC DNA (Fig. 2). This is in contrast to what was observed in the BAC clones of other plant species such as rice (Zhang et al. 1996a) or sorghum (Woo et al. 1994), in which each BAC clone showed several restriction fragments after *Not*I digestion. This seems to indicate that the *Not*I restriction sites are less common in the *Citrus* and *Poncirus* genomes.

Library screening with restriction fragment length polymorphic (RFLP) markers linked to *Ctv*

To evaluate further the quality of the library, we screened it with four RFLP markers that were located in the *Ctv* gene region. The 24,576 clones of the library were double-spotted onto 16 nylon membrane filters, and a colony hybridization procedure was followed to identity positive BAC clones. The four RFLP probes used in library screening were derived from randomly amplified polymorphic DNA (RAPD) fragments identified in the genetic mapping of the *Ctv* gene (unpublished data). The corresponding fragments (see below) for Z16#2, AD08#1, and C19-G5 had also been cloned and characterized previously by Fang et al. (1998) in their work on *Ctv* mapping. Genomic Southern hybridization performed by

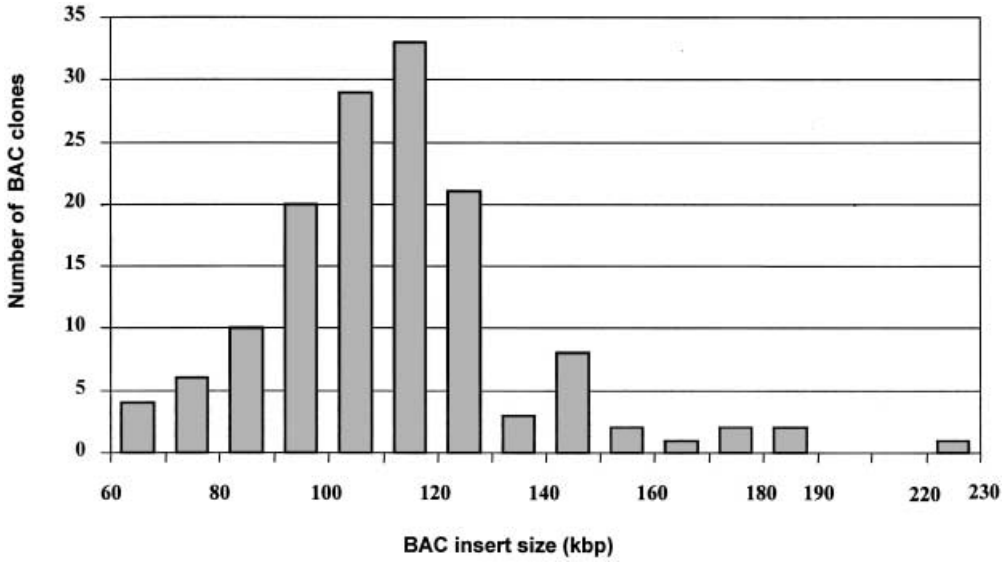

Fig. 1 Insert size distribution of clones in the *Bam*HI citrus BAC library. BAC inserts were released by digestion of DNA minipreps with *Not*I, fractionated on a pulsed-field gel, and estimated for sizes by comparison with lambda concatemer size markers

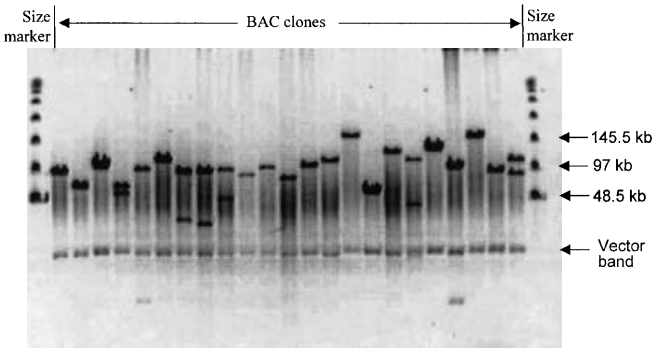

Fig. 2 Negative image of an ethidium bromide-stained pulsed-field gel showing the inserts of some randomly selected citrus BAC clones released by *Not*I restriction enzyme. The *two outside lanes* are molecular size markers (lambda concatemers from Sigma Chemical, St. Louis, Mo.)

Table 1 Positive BAC clones identified with four DNA probes linked to the CTV resistance gene. All probes were derived from cloned RAPD fragments

Probe	Number of hits	Positive clones (insert sizes)
Z16#2	4	BC5 (75 kb), BC21 (175 kb), BC31 (175 kb), BC33 (105 kb)
AD08#1	8	BC5 (75 kb), BC9 (135 kb), BC19 (100 kb), BC21 (175 kb), BC31 (175 kb), BC33 (105 kb), BC17 (110 kb), RB116
C19-G5	7	RB6 (75 kb), RB71 (125 kb), RB80 (95 kb), RB98 (105 kb), RB119 (150 kb), RB57 (105 kb), RB100 (85 kb)
AM02-d5	5	RB48 (95 kb), RB69 (100 kb), RB102 (95 kb), RB104 (115 kb), RB122 (120 kb)

Fang et al. (1998) and ourselves showed that the Z16#2 (RfZ16) probe detected one fragment in the *Poncirus* genome and no fragments in the *Citrus* genome. Probes AD08#1 (RfAD08) and C19-G5 (RfC19) are single-copy sequences in both *Citrus* and *Poncirus* genomes. Probe AM02-d5 was derived from RAPD marker OPAM02 that was mapped close to *Ctv* (unpublished); it is a single-copy DNA fragment in both *Citrus* and *Poncirus* genome. Table 1 shows the results of library screening with these markers. Each of the probes detected four to eight positive clones; their inserts ranged from 75 kb to 175 kb, with an average size of 115 kb, which seems to agree well with the library's estimated representation based on the average insert size and the total number of clones.

Identification of BACs containing RGCs

Previously, 22 genomic sequences that shared strong similarity to the NBS-LRR class of plant *R* genes were cloned (Deng et al. 2000). Based on their amino acid identities, they were grouped into ten classes, designated as RGC1–RGC10. To facilitate identification of RGC-containing BACs, we selected six representative clones from classes RGC1–5 (clones Pt3, Pt6, Pt7, Pt14, Pt18, 11P31) and another seven representative clones from classes RGC6–10 (clones Pt8, Pt9, Pt19, 18P33, 18P34, 16R1–13, and 16R1–19), pooled these to make two mixed probes, NBSmp1 and NBSmp2, and used them to screen the BAC library. Probe pool NBSmp1 identified 236 clones, while NBSmp2 hybridized with 118 clones. The majority of these clones hybridized with one probe pool or the other, but 32 out of the total 322 clones (9.9%) hybridized with both probe pools, though showing different strength of hybridization signals between pools.

The copy numbers of NBS sequences in some of these RGC BAC clones were estimated. Eighteen BAC clones identified with the NBSmp1 and 11 BAC clones identified with the NBSmp2 were selected for this analysis. Together, they represented 19 RGC BAC contigs and ten RGC BAC singletons (see below and Table 2). The

Table 2 Citrus RGC BAC contigs. The RGC BAC clones were identified using two probe pools (NBSmp1 and NBSmp2) in library screening and assembled into contigs using computer program FPC, based on BAC fingerprints on polyacrylamide sequencing gels. Probes NBSmp1 and NBSmp2 were made by pooling six citrus RGC clones (Pt3, Pt6, Pt7, Pt14, Pt18, and 11P31) or another seven citrus RGC clones (Pt8, Pt9, Pt19, 18P33, 18P34, 16R1–13, and 16R1–19), respectively. All 13 RGCs were cloned previously and showed similarities to the NBS-LRR class of plant disease resistance genes. Boldfaced and underlined clones were characterized by Southern analysis (see text); contig sizes (in kb) were estimated

Contig name	BAC clones	Probes	Size (kb)
NBS Ctg1	**RB1**, RB7, RB21, RB44, RB47	NBSmp2	170
NBS Ctg2	**RB2**, RB24, RB101, RB121, RB123	NBSmp1	180
NBS Ctg3	**RB3**, RB60, RB63, RB64, RB111	NBSmp1	154
NBS Ctg4	**RB8**, RB13, RB38, RB52, RB53, RB66, RB82, RB83, RB92, RB97, RB117	NBSmp2	197
NBS Ctg5	**RB11**, RB26	NBSmp1	149
NBS Ctg6	**RB14**, RB35	NBSmp2	120
NBS Ctg7	RB15, RB18, RB120	NBSmp2	120
NBS Ctg8	RB16, RB115	NBSmp1	130
NBS Ctg9	**RB17**, RB77, RB93	NBSmp1	146
NBS Ctg10	**RB25**, RB94, RB95	NBSmp1	140
NBS Ctg11	**RB27**, RB51, RB106, RB111, RB128	NBSmp1	178
NBS Ctg12	**RB28**, RB37, RB124	NBSmp1	200
NBS Ctg13	**RB33**, RB58, RB107	NBSmp2	120
NBS Ctg14	RB42, **RB103**, RB105, RB131	NBSmp2	120
NBS Ctg15	**RB45**, RB81	NBSmp1	173
NBS Ctg16	**RB46**, RB76, RB79, RB90	NBSmp1	144
NBS Ctg17	RB49, RB125	NBSmp1	120
NBS Ctg18	**RB54**, RB74, RB88, RB95, RB112, RB127, RB130	NBSmp1	210
NBS Ctg19	RB29, RB55, RB61	NBSmp2	132
NBS Ctg20	RB59, RB109	NBSmp1	123
NBS Ctg21	**RB65**, RB67	NBSmp1	200
NBS Ctg22	**RB70**, RB75	NBSmp1	168
NBS Ctg23	**RB73**, RB133	NBSmp2	173
NBS Ctg24	RB5, RB9, RB22, RB40, **RB84**	NBSmp2	140
NBS Ctg25	RB43, RB85, RB87, RB89	NBSmp2	190
Singletons	**RB30**, **RB32**, **RB50**, **RB56**, **RB78**, **RB129**, **RB19**, RB62, RB68, RB72	NBSmp1	
Singletons	**RB4**, RB12, **RB23**, RB36, **RB91**, **RB114**	NBSmp2	

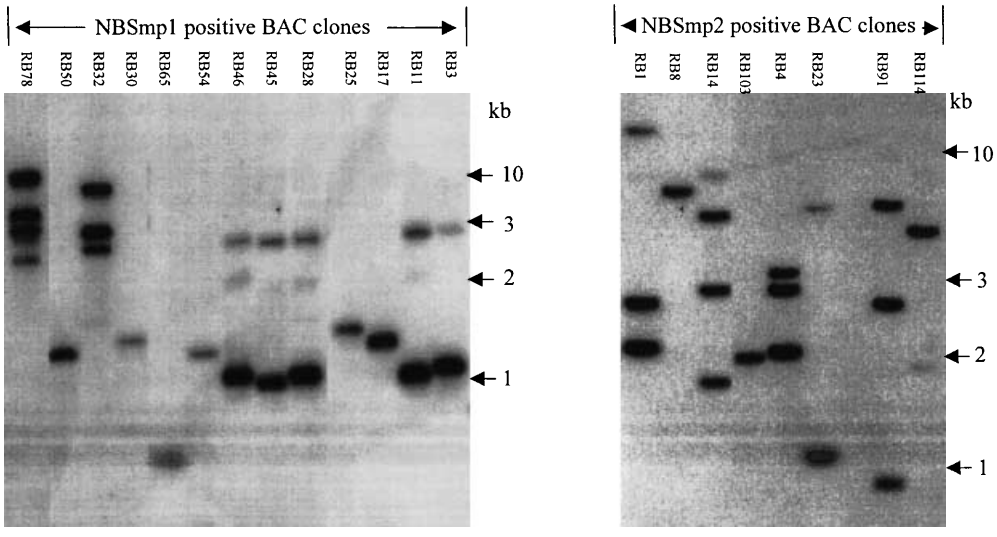

Fig. 3 Autoradiographs of 13 NBSmp1 and 8 NBSmp2 positive BAC clones probed with the original probe pools used in library screening. BAC DNA of the NBSmp1 positive clones was digested with *Bam*HI and *Hin*dIII, while the DNA of the NBSmp2 positive clones was digested with only *Hin*dIII. The NBSmp1 and NBSmp2 probe pools consisted of six clones (Pt3, Pt6, Pt7, Pt14, Pt18, and 11P31) from classes RGC1–5 and seven clones (Pt8, Pt9, Pt19, 18P33, 18P34, 16R1–13, 16R1–19) from classes RGC6–10, respectively. Post-hybridization wash conditions consisted of two washes at 65°C with 0.5× SSC+0.1% SDS (20 min/each time), and one wash at 65°C with 0.2× SSC+0.1% SDS. *Numbers* beside the autoradiographs are molecular weight markers (in kilobases)

BAC DNA of these clones was digested with *Hin*dIII and/or *Bam*HI, fractionated on agarose gels, blotted onto nylon membranes, and probed with the NBSmp1 or NBSmp2 probe pools. Under a stringent wash condition (0.2× SSC, 0.1% SDS, at 65°C), the probe pool NBSmp1 detected one to four fragments in each of the 18 NBSmp1-positive BACs, with an average of 1.7 fragments per BAC; the sizes of the hybridized fragments ranged from 0.8 kb to 10 kb. Under the same wash condition, the probe pool NBSmp2 detected one to four fragments

in each of the 11 NBSmp2-positive BACs and an average of 2.5 fragments per BAC; the RGC-containing fragments were 0.5 kb to 15 kb in size (Fig. 3). Nine out of the eighteen NBSmp1 positive BAC clones and five out of the eleven NBSmp2 positive BAC clones each contained two or more copies of RGC sequences.

Development of RGC BAC contigs

The DNA sequencing gel-based fingerprinting (Zhang and Tao, personal communication) and computer-aided contig assembly (Soderlund et al. 1997) approaches were used to determine the relationship among the RGC-containing BAC clones identified above and to construct BAC contigs. These techniques have been used in construction of megabase-size BAC contigs (Marra et al. 1997) and genome-wide physical mapping (Zhang and Tao, personal communication). In the present study, 61 NBSmp1 positive clones and 48 NBSmp2 positive clones were fingerprinted using the sequencing gel-based procedure. The resulting fingerprints were then analyzed using the FPC computer program (Soderlund et al. 1997) to construct contigs. A number of Southern hybridization experiments were performed with some of the fingerprinted clones to determine the appropriate cut-off value settings for use in the FPC program. Results indicated that a cut-off value of $1e^{-20}$ seemed to be suitable for the present study (data not shown). With this setting, 15 contigs were assembled from 49 of the 61 NBSmp1 positive clones and 10 contigs from 42 of the 48 NBSmp2 positive clones (Table 2). Southern analysis indicated that BAC clones from different assembled contigs showed different hybridization profiles (Fig. 3). A number of clones were left as singletons (Table 2) due to insufficient numbers of common fingerprint bands with other fingerprinted clones. The assembled contigs consist of 2–11 BACs and are estimated to have a length of 120–250 kb (Table 2).

Discussion

We have been attempting to isolate the *Ctv* gene from *P. trifoliata* using a map-based cloning strategy. Construction of BAC libraries is an important component of this effort. Compared to the yeast artificial chromosome (YAC) system (Burke et al. 1987), the BAC system offers a number of advantages (Shizuya et al. 1992) for large DNA fragment cloning, but the development of BAC libraries with acceptable average insert sizes has been demanding technically and can be a rate-limiting step in map-based gene cloning projects. For cloning the *Ctv* gene, we previously constructed a *Hin*dIII BAC library, but the average insert size of the clones was about 80 kb (Gmitter et al. 1998). Consequently, chromosome walking using the library was slow and difficult. The current library consists of more than 24000 clones with inserts ranging from 60 kb to 230 kb, thus representing approximately seven citrus haploid genome equivalents. The improved genome coverage and the greater than 40% increase in average insert size, compared to the previous library, have facilitated chromosome walking in and BAC contig development for the *Ctv* gene region. When the library was screened with four *Ctv*-linked probes, four to eight positive clones were identified by each probe. The insert sizes of these positive clones were 75–175 kb with an average of 115 kb. Chromosome walking was initiated using the insert ends of these BAC clones. In each case of library screening with the BAC insert ends, several clone hits were observed. Thus, by using this library we were able to construct a BAC contig of several hundred kilobases that covers the *Ctv* region (unpublished data).

Although this *Bam*HI library was constructed originally for *Ctv* positional cloning, we envisage many other uses for it. In addition to the CTV resistance gene, USDA17–47 also harbors several other resistance genes including *Tyr1*, the major gene responsible for citrus nematode (*Tylenchulus semipenetrans*) resistance (Ling et al. 2000) and genes for *Phytophothora* resistance. These resistance genes should be represented in the library according to its genome coverage; we expect that this BAC library would be useful in cloning these resistance genes when high-resolution maps of the gene regions become available. The library may be also useful to isolate the genomic sequences for genes responsible for other horticulturally important traits like cold tolerance and polyembryony.

Among the plant disease resistance genes cloned so far, the majority of them belong to the NBS-LRR class (Hammond-Kosack and Jones 1997). Based on similarity inferences, their protein products seem to be involved in signal transduction pathways (Hammond-Kosack and Jones 1997). Recently, numerous resistance gene-like NBS sequences have been isolated from a number of plant species using a PCR approach with degenerate primers based from the cloned NBS-LRR class of *R* genes (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). Many of these sequences have been shown to be associated with resistance gene loci (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996), and at least some of them seem to be part of disease resistance genes (Meyers et al. 1998). It is estimated that NBS sequences account for nearly 1% of the total genome sequences in *Arabidopsis* and that the rice genome contains over 700 copies of NBS type sequences (Meyers et al. 1999). Disease resistance genes in citrus have not been located or mapped until recently; molecular cloning of citrus *R* genes has focused on *Ctv* (Deng et al. 1997; Fang et al. 1998; Gmitter et al. 1996; Mestre et al. 1997). Consequently, the nature, copy number, and genomic distribution features of citrus *R* genes are poorly understood. In genetic mapping of *Ctv* and *Tyr1*, we recognized that these two genes are closely linked and that there exists a cluster of resistance gene sequences in the *Ctv-Tyr1* region, which extends some 12 cM. The finding of this re-

sistance gene cluster was based on DNA marker analysis and genetic mapping data, rather than on any physical evidence (Deng et al. 2000).

Using the above-mentioned PCR approach, Deng et al. (2000) identified ten classes (RGC1–RGC10) of citrus RGC sequences similar to the NBS-LRR class of *R* genes. When the *Bam*HI library was screened with 13 representative citrus RGC sequences from the ten classes, 322 hybridizing BAC clones were identified. This number accounts for nearly 1.2% of the clones in the library. The upstream and/or downstream sequences of the NBS regions in some of these RGC BACs have been obtained, and the sequences were found to carry the other characteristic motifs (i.e. LRRs) of the NBS-LRR class of resistance genes (unpublished data). These data suggest that some RGCs may be actual citrus *R* genes. Therefore, characterization of these RGC BAC clones may provide some insight regarding citrus *R* genes before they are cloned in full-length. One of these features found is the clustering of citrus NBS-type RGCs. When 29 BACs from 19 RGC BAC contigs and ten unassembled RGC BACs were digested with *Hin*dIII and/or *Bam*HI and re-probed with the probes used in the initial library screening, more than 40% of the BAC clones showed two or more hybridizing fragments (up to 4) under stringent wash conditions (0.2× SSC, 65°C). Therefore, it is likely that two or more copies of NBS sequences are clustered in each of these BAC clones. This clustering feature of RGCs may be a good indication of *R* gene distribution in the citrus genome. Clustering of *R* genes has been well-documented in several model plant species based on genetic analysis and on recent analysis of large-insert clones or BAC contigs (Meyers et al. 1998). Sometimes resistance gene clusters span many megabase regions (Meyers et al. 1998). The availability of a citrus BAC library (in particular assembled BAC contigs) will be of value in elucidating the organization and evolution of citrus *R* genes.

Twenty-five BAC contigs were assembled by FPC out of 109 fingerprinted RGC BAC clones. Based on the library's genome coverage (7×), we anticipate that the 322 RGC BAC clones might result in 40–70 BAC contigs. For simplicity, it seems reasonable to assume that each of the contigs represent one genetic locus; therefore, the 322 RGC BAC clones may correspond to 40–70 genetic loci in the citrus genome. Assuming that on average two copies of NBS sequences are contained in each BAC according to the data from the 29 characterized RGC BAC clones, we expect that 80–140 unique NBS sequences may be found in the 322 RGC BAC clones. These NBS sequences in the RGC BAC clones may provide a good starting point for mapping and cloning citrus *R* genes in the future, if the majority of citrus *R* genes belong to the NBS-LRR class, as in other plant species (Meyers et al. 1999). Some of the insert ends of the RGC BAC clones are being isolated for future use as DNA markers. If any of these ends is closely linked to *R* genes and the BAC contigs cover the target gene regions, then isolation of the *R* genes may not require tedious and time-consuming marker identification and BAC contig development. Therefore, citrus *R* gene cloning should be facilitated.

Large-insert DNA clones are essential for many genomic studies including physical mapping of genomic regions, specific chromosomes, or the whole genomes of a species; analysis of genome organization and evolution of complex genes or multi-gene families; and establishment of the relationship between genetic and physical distances. Because of the nature of the DNA source used in the library construction, we expect that both the *Citrus* and *Poncirus* genomes would be represented in the library and, therefore, envisage that the library will provide a very useful resource for studies of both genomes in several aspects.

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