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ORIGINAL PAPER

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Construction of a *Hin*dIII Bacterial Artificial Chromosome library and its use in identification of clones associated with disease resistance in chickpea

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Abstract A chickpea (*Cicer arietinum* L.) Bacterial Artificial Chromosome (BAC) library from germplasm line, FLIP 84-92C, was constructed to facilitate positional cloning of disease resistance genes and physical mapping of the genome. The BAC library has 23,780 colonies and was calculated to comprise approximately 3.8 haploid-genome equivalents. Studies on 120 randomly chosen clones revealed an average insert size of 100 kb and no empty clones. Colony hybridization using the RUBP carboxylase large subunit as a probe resulted in a very low percentage of chloroplast DNA contamination. Two clones with a combined insert size of 200 kb were isolated after the library was screened with a Sequence Tagged Microsatellite Site (STMS) marker, Ta96, which is tightly linked to a gene (*Foc*3) for resistance to fusarium wilt caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) race 3 at a genetic distance of 1 cM. Also, these two clones were analyzed with several resistance gene analog (RGA) markers. End sequencing of these clones did not identify repetitive sequences. The development of the BAC library will facilitate isolation of *Foc3* and allow us to perform physical mapping of this genomic region where additional R genes against other races of the wilt causing pathogen are positioned.

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Introduction

Chickpea is a cool-season food legume and an economically important crop worldwide. Genomics is a new field of research in chickpea, and hence several opportunities can be exploited. Chickpea genome analysis to date has involved linkage map development with 354 markers covering 2,077.9 cM at an average distance of 6.8 cM between markers (Winter et al. 2000). There are additional linkage maps available that have been developed from different segregating populations of chickpea (Santra et al. 2000; Tekeoglu et al. 2002; Flandez-Galvez et al. 2003). Combining these maps into a consensus linkage map is in progress (International Chickpea Genome Consortium 2003). In most of the maps, disease resistance genes share a common position. These observations provide the necessary framework for physical mapping and isolation of disease resistance genes by map-based cloning. One essential tool to fulfill this objective is the availability of large insert genomic libraries (Yu et al. 2000). These libraries have been used to develop detailed genetic and physical maps of major crops and for positional cloning of genes of interest (Zhang and Wing 1997; Stein et al. 2000). BAC libraries are preferred over Yeast Artificial Chromosome (YAC) libraries for various reasons, e.g. BAC DNA is easy to purify in contrast to YAC DNA. Also BAC clones are stable in host strains, physical maps can be constructed, and there is minimal chimerism. One of the major applications of the BAC library is to physically map gene-rich regions. Since 1994, BAC libraries have been available for many crops including sorghum (Woo et al. 1994), *Arabidopsis* (Choi et al. 1995), apple (Vinatzer et al. 1998), wheat (Moullet et al. 1999), soybean (Meksem et al. 2000) and pea (Coyne et al. 2000).

Chickpea has a relatively small genome when compared to other cool-season legumes, with an estimated size of 740 Mb (Arumuganathan and Earle 1991) and has a short "seed to seed" period (3–4 months). Based on these considerations, the enormous usefulness of a BAC library in genome characterization, we undertook the

formation of a chickpea BAC library for use our laboratory and the International chickpea research community. Initial screening of the library with the DNA markers tightly linked to resistance genes demonstrates the utility of this essential tool for positional cloning of important genes in chickpea.

A STMS marker, Ta96, was recently shown to be tightly linked to the gene for resistance to fusarium wilt caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) race 3 at a distance of 1 cM (Dev et al. 2003; manuscript in communication). In this paper, we explain the construction of a BAC library from FLIP 84-92C, a chickpea germplasm accession (Rajesh et al. 2002a), and its use in identifying clones associated with disease resistance by screening with Ta96.

Materials and methods

Pre-treatment of the plant material

Chickpea germplasm line FLIP 84-92C was used to construct the *Hin*dIII BAC library. The seeds of FLIP84-92C were obtained from the International Center for Agricultural Research in the Dry Area (ICARDA) and grown in a growth chamber in perlite medium for 14 days, with a cycle of 16-h light and 8-h darkness at 22°C. Two weeks after germination, seedlings were grown in continuous darkness for 3 days to reduce the carbohydrate content of the leaves.

High-molecular-weight (HMW) DNA isolation

Nuclei were isolated from 25 g of leaves (Zhang et al. 1995). The frozen tissue was ground into a powder in liquid nitrogen and transferred into an ice-cold beaker containing 200 ml of homogenization buffer (10×HB=0.1 M Tris, 0.8 M KCl, 0.1 M EDTA, 10 mM of Spermidine, 10 mM of Spermine, pH 9.4) + 300 μl β-mercaptoethanol and the contents were allowed to mix well. The slurry was filtered using miracloth and centrifuged in a swing bucket rotor at 3,600 g at 4°C for 20 min (Eppendorff, USA). The supernatant was discarded and the pellet was mixed with 1 ml of ice-cold wash buffer (HB + 0.5% Triton X-100 with no β-mercaptoethanol). 20% Triton X-100 stock was prepared by mixing Triton X-100 in HB without β-mercaptoethanol and stored at 4°C. The pellets were then gently suspended in 30 ml of this buffer using a paintbrush. The nuclei were re-suspended and washed twice by centrifuging at 3,600 g at 4°C with the wash buffer. After the final wash, the pellet was suspended in 1 ml of HB without β-mercaptoethanol using a paintbrush.

Embedding the nuclei in agarose plugs

One percent-low-melting point (LMP) agarose was prepared in HB without β-mercaptoethanol and was stored in a 45°C water bath. The nuclei were pre-warmed at 45°C and mixed with an equal volume of pre-warmed 1% LMP agarose using a cut-off pipette tip. The mixture was aliquotted into ice-cold plug molds on ice. After the agarose was completely solidified, the plugs were transferred into 5–10 vol of lysis buffer (0.5 M EDTA, pH 9.0–9.3, 1% sodium lauryl sarcosine and 0.1% proteinase K). The buffer was added just before transferring the plugs to lysis buffer. The plugs were incubated for 24–48 h at 50°C with gentle shaking, then washed once with 0.5 M EDTA, pH 9.0–9.3 for 1 h at 50°C, once with 0.05 M EDTA, pH 8.0, for 1 h on ice and finally stored in 0.05 M EDTA, pH 8.0 at 4°C.

History of the vector

The BAC vectors are derived from the *Eschericha coli* "F" factor plasmid, which contains genes for strict copy number control and unidirectional DNA replication. The BAC vector pCLDO4541 (V41) was used for the large insert library construction. V41 is 27.6 kb in size and derived from the plasmid vector pRK290 of 20 kb, which is a derivative of a native plasmid RK2 (Ditta et al. 1980). RK2 belongs to the P1 incompatibility group and has a size of 56 Kb. The genes oriV, trfA and trfB constitute the replicons of RK2 and its derivative pRK290, which exist as 5–8 copies/chromosomal equivalent in *E. coli*. V41 is a binary vector for *Agrobacterium*-mediated plant transformation and has been shown to be capable of stable maintenance of the large plant DNA (Moullet et al. 1999).

Vector preparation

V41 was isolated using the Qiagen Large Construct Kit followed by cesium chloride/ethidium bromide equilibrium centrifugation at 90,000 rpm for 3 h at 20°C. After isolation, the vector DNA was digested to completion with the *Hin*dIII restriction enzyme overnight at 37°C and assayed by gel electrophoresis. After a chloroform/isoamyl alcohol (IAA) extraction and an ethanol precipitation, the vector was de-phosphorylated with 0.25 U of calf intestinal alkaline phosphatase (CIAP) (BRL, USA) per microgram of DNA for 30 min at 37°C. CIAP was heat-inactivated at 75°C for 10 min in the presence of 100 mM of NaCl, 1 mM of EDTA and 0.25% SDS. The DNA was extracted with chloroform/IAA, precipitated with ethanol and re-suspended in TE, pH 8.0, at a concentration of 20 ng/μl, and directly used in the ligation reaction. The efficiency of de-phosphorylation was tested by self-ligation of the BAC vector. Self-ligation of non-de-phosphorylated BAC vector was used as a control. Only those preparations in which de-phosphorylation resulted in more than 98% reduction in the number of transformants were used for construction of the BAC library. The cloning efficiency of vector preparation was tested by ligation with Lambda *Hin*dIII; only those preparations that resulted in a minimum of 50% white colonies were used for library construction.

Partial digestion and size selection of HMW DNA

Each agarose plug that contained about 1 μg of DNA was cut into 12 equal pieces. The amount of the enzyme *Hin*dIII (Gibco-BRL) added to each plug ranged from 0.1–2 units per μg of DNA and the digestion time was extended to 20 min at 37°C. Each digestion was carried out in a 200-μl total volume and the reactions were stopped by adding 20 μl of ice-cold 0.5 M EDTA, pH 8, on ice. Partially digested DNA was fractionated on a 1% LMP agarose gel in 0.5×TBE (45 mM of Tris-borate, pH 8, 1 mM of EDTA) by Pulsed Field Gel Electrophoresis (PFGE) at 6 V/cm, and 10–60 s switch time for 16 h at 11°C (CHEF DRIII system, Bio Rad, USA). DNA fractions ranging from 100 kb to 200 kb were excised from the gel and directly used for the second size selection in 0.8% LMP agarose using the following conditions: 4 V/cm, 5 s switch time, 9 h at 11°C, and 0.5×TBE). The agarose slices were equilibrated for 1 h, on ice, in 10 mM Tris-HCl, pH 7.5, 1 mM of EDTA and 40 mM of NaCl with one change of buffer after 30 min, melted at 68°C for 5 min and digested with 1 U of agarase (Epicenter, USA) per 100 mg of LMP-agarose for 30 min at 45°C. The enzyme was heat-inactivated at 70°C for 5 min and the DNA solution was used directly for the ligation reaction. Woo et al. (1994) found that it was essential to perform two such size selections to increase the average insert size of BACs and eliminate small DNA fragments trapped after the first size selection.

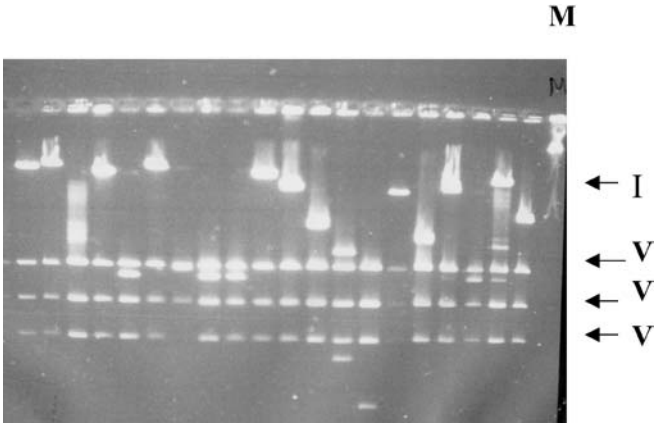

Fig. 1 Insert size determination. *V*—Vector; *I*—Insert; *M*—Lambda-ladder PFG Marker

Ligation, transformation and selection

The ligation was carried out in a 100–150 μl vol in which about 60–120 ng of *Hin*dIII, partially digested and size-selected chickpea DNA, was ligated to 200–400 ng of vector (molar ratio of about 1:4 with a vector excess) with 6 U of T4 DNA ligase (Gibco BRL, USA) and incubated overnight at 16°C.

The recombinant vectors were introduced into ElectroMAX DH10B cells (Gibco-BRL, USA) by electroporation using a Cell Porator and a Voltage Booster system (Gibco-BRL, USA). A 1.5-μl aliquot of ligation mixture was added to 18 μl of electrocompetent *E. coli* cells for a single electroporation. The Cell Porator settings were 350 V, 330 μF capacitance, low ohms impedance and fast charge rate, and the Voltage Booster setting was 4 k-ohms resistance. After electroporation, the cells were transferred to 1 ml SOC solution (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM of NaCl, 2.5 mM of KCl, 10 mM of $MgCl_2$, 10 mM of $MgSO_4$, 20 mM of glucose, pH 7.0) and incubated at 37°C with shaking at 200 rpm for 20 min. The cells were spread on Luria Bertani (LB) plates containing tetracycline (15 μg/ml), X-gal (30 μg/ml) and isopropylthio-β-D-galactoside (20 μg/ml), grown at 37°C for 20 h and stored at 4°C. White colonies containing chickpea DNA inserts were transferred to 384-well microtiter plates (Medos, Australia) containing 50 μl of LB-freezing buffer (9:1) (36 mM of K_2HPO_4, 13.2 mM of KH_2PO_4, 1.7 mM of citrate, 0.4 mM of $MgSO_4$, 6.8 mM of $(NH_4)_2SO_4$, 4.4% glycerol, 15 μg/ml of tetracycline, LB) per well, with the help of Flexy's Bio robot, incubated at 37°C for 24 h and stored at −80°C.

Isolation of recombinant DNA and insert analysis

Clones were streaked onto LB plates containing 15 μg/ml of tetracycline. One hundred and twenty random single colonies were picked up and grown in 5 ml of LB with antibiotic (15 μg/ml of tetracycline) at 37°C for 20 h. The alkaline lysis method (Sambrook et al. 1989) was used for preparing the plasmid DNA. Samples were digested with *Not*I (NEB, USA) overnight and then loaded on a 0.8% agarose gel. The gel was subjected to PFGE overnight at 6 V/cm in 0.5×TBE at 16°C using a 1–12 s discontinuous ramped switch time. The average insert size of the BAC library was estimated to be 100 kb by comparison with a lambda mid-range size standard (NEB, USA) (Fig. 1).

Filter preparation

High-density filters were made by replicating twentyfour 384-well microtiter plates onto single pre-wet Hybond N+ filters (8×12 cm) (Amersham, USA) placed on LB/agar plate containing 15 μg/ml of tetracycline using Flexy's Bio-robot. Each sample was double-spotted for confirmation of the hybridization. Filters were incubated overnight on the same medium. The fixation of plasmid DNA to the filter was performed according to the filter manufacturer's recommendations.

Quantitation of chloroplast DNA contamination in the BAC library

Since the BAC library was constructed from leaf DNA, clones containing chloroplast DNA were expected to be present in the BAC library. In order to estimate the percentage of BAC clones containing chloroplast DNA, the filters representing the entire chickpea BAC library were hybridized with the barley Kfp-231 chloroplast DNA probe, encoding the RUBP carboxylase large subunit.

BAC screening using Ta96

Ta96 was identified as being a closely linked marker to *Foc* 3 at a genetic distance of 1 cM (Dev et al. 2003; manuscript in communication). During the primary screening, 62 super pools of each 384-well BAC plates were made and were screened with Ta96, an STMS marker, using PCR. Secondary screening was performed by making pools of each row of positive plates identified during the primary screening, which was followed by screening individual clones of positive rows as tertiary screening. As control reactions, parental lines were also amplified with this marker along with the BAC clones. The amplification pattern of Ta 96 was shown in Fig. 2. Experimental details are as described in Rajesh et al. (2002b). These clones were digested with *Hin*dIII and were fingerprinted (Fig. 3).

Screening of BAC clones using RGA primers

The BAC clones were screened with the following RGA primer pairs: S1-GGTGGGGTTGGGAAGACAACG, AS1-CAACGCTAGTGGCAATCC; S2-GGIGGIGTIGGIAAIACIAC, AS3-IAGIGCIAGIGGIAGICC; NPLOOP-TCAATTAATGTTTGAGTTATTTGTA, Nkin2-GTAACTAAGGATAGA; XLRR For-CCGTTGGACAGGAAGGAG, XLRR Rev-CCCATAGACCGGACTGTT; Pto kin1-GCATTGGAACAAGGTGAA; Pto kin2-AGGGGGACCACCACGTAG; LM637-ARIGCTARIGGIARICC, LM638-GGIGGIGTIGGIAAIACIAC; Cre3Ploop: GCGGGTCTGGGAAATCTACC, Cre3-k3-CTGCAGTAAGCAAAGCAACG; Xa1NBS-GGCAATGGAGGGATAGG; Xa1NBS-R-CTCTGTATACGAGTTGTC. Reaction conditions were as described in Rajesh et al. (2002c).

BAC end-sequencing and contig development

BAC DNAs were purified using the BAC_{96} Miniprep kit (Millipore, USA). The primers for end-sequencing were designed from the flanking region of the *Hin*dIII cloning site and synthesized by MWG, USA. The sequences of the primers are: V41F-GCGATTAAGTTGGGTAACGC and V41R-CACAGGAAACAGCTATGACC. One and a half micrograms of clean BAC DNA was used for direct PCR cycle sequencing with extended (66) cycles on an ABI PRISM 377 automatic sequencer (Applied Biosystems, USA). Sequence comparison was carried out using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

For contig development, the PCR primers were designed from end-sequences using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by MWG, USA (Table 1). PCR reactions were carried out in a total volume of 50 μl containing 1 × reaction buffer containing 1.5 mM of $MgCl_2$, each dNTP at 0.2 mM, 1 U of *Taq* polymerase (Promega), 1 μM of each primer and 30 ng of BAC DNA. PCR conditions were as follows: initial denaturation at 94°C for 5 min,

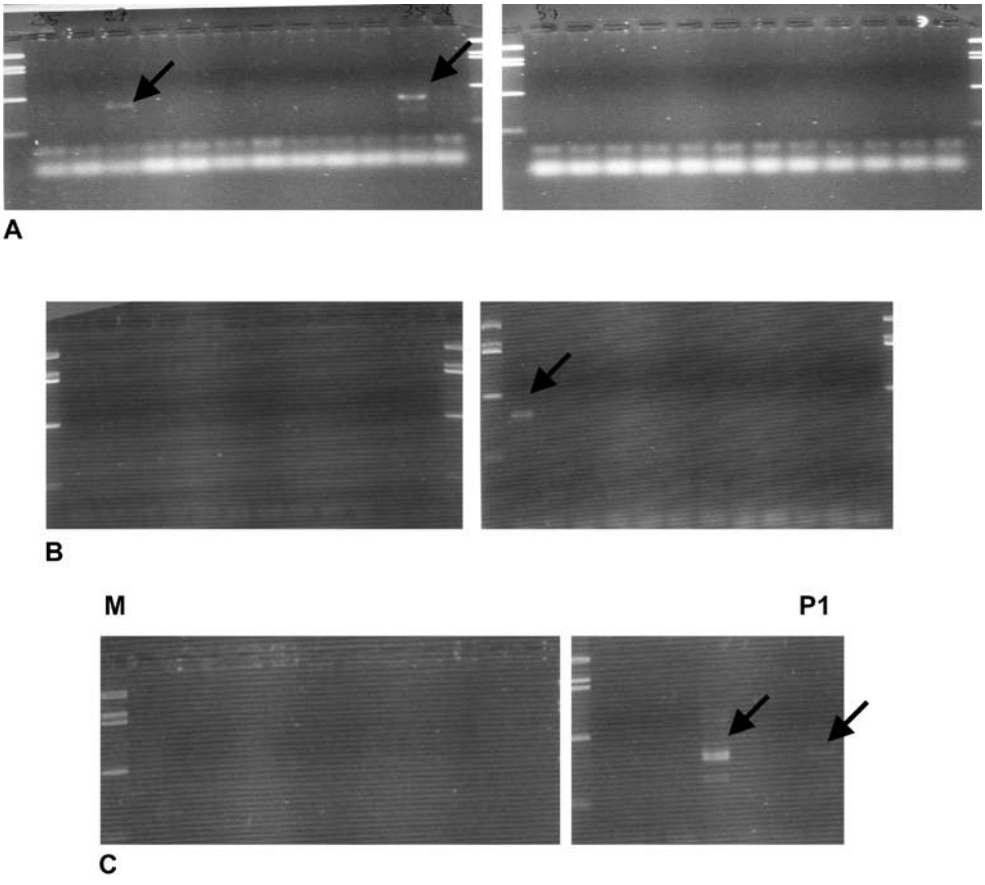

Fig. 2 BAC library screening using STMS primer Ta96. **A** Primary screening—PCR amplification of bulks of each 384-well plate. **B** Secondary screening—bulks of individual columns of the positive plate. **C** Tertiary screening—individual clones of the positive column. *M*—Lambda Bst-N1 marker, P1-FLIP84-92C. The *arrows* represent the band amplified by Ta96

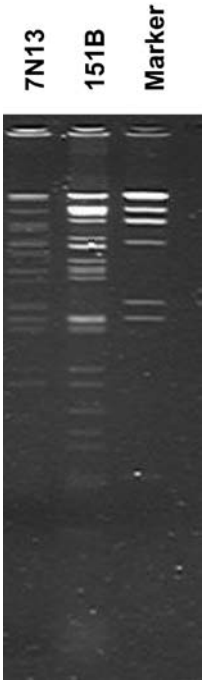

Fig. 3 BAC clones digested with *Hin*dIII; the marker is phage lambda DNA digested with *Hin*dIII. An ethidium bromide-stained agarose gel

Table 1 Sequences of BAC end-primers

Primer name	Sequences
15B1(L)-Forward	ACTCACCACCATGTCCCTTC
15B1(L)-Reverse	AACACGGGTGTGGCAACT
15B1(R)-Forward	TGCATTGAGCTCCCTCATAA
15B1(R)-Reverse	TACTCAACCAAATGCCGTGA
7N13(L)-Forward	AGCCCTTTTATGCCTGATCC
7N13(L)-Reverse	AAGCCTTGGTTTCTTGGACA
7N13(R)-Forward	CGATAAGCTTAGCATCTGCTTG
7N13(R)-Reverse	CCATCCCCAAATTCTCAAAA

followed by 40 cycles of 20 s at 94°C, 1 min at 57°C and 2 min at 72°C. Final elongation was carried out at 72°C for 8 min. The PCR products were run on a 2% 1 × TBE agarose gel (Fig. 4)

Results and discussion

BAC library construction and characterization

The development and characterization of a high quality BAC library for chickpea germplasm accession FLIP 84-92C has been described. High-molecular-weight (HMW) DNA and of high quality, are prerequisites for BAC library construction. The nuclei method was used to isolate HMW DNA in chickpea in which nuclei were embedded in agarose plugs. The agarose acts as a solid

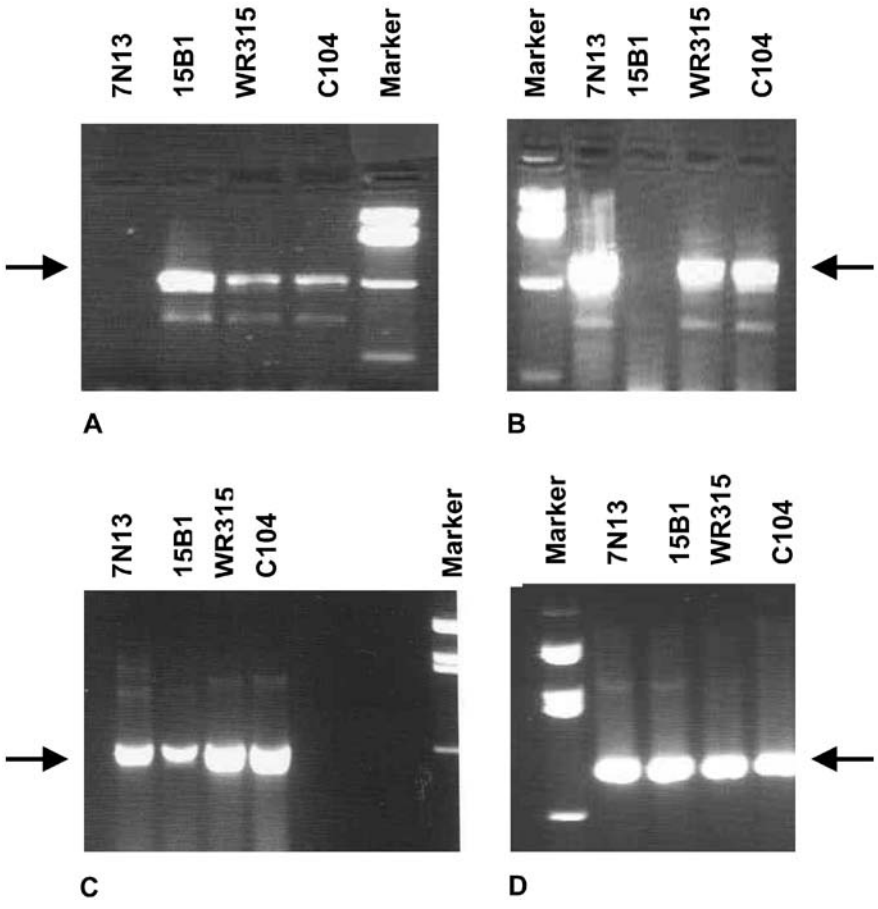

Fig. 4 Amplification pattern of the BAC clones using primers designed from end-sequences. **A** Primer 15B1(*L*). **B** Primer 7N13 (*R*). **C** Primer 7N13 (*L*). **D** Primer 15B1 (*R*). *Marker*—Lambda Bst-N1 marker. WR315 and C104 are the parental lines used for mapping Ta96. The *arrows* indicate the bands amplified by these primers

yet porous matrix which allows the diffusion of various reagents for DNA purification and subsequent manipulations, while preventing the DNA from being sheared (Schwartz and Cantor 1984). This method has worked well for several divergent plant taxa, and also is simple and cost-effective (Zhang et al.1995). High-molecular-weight DNA ranging between 100 to 200 kb was used during size selection. Previous results in other systems have shown that recombinant BACs larger than 350 kb have not been recovered. One possible reason for the inability to recover larger BACs was that there might be a limit to the size of a molecule that can be delivered into *E.coli* by electroporation.

For library construction, the transformation efficiency of the host cells is critical. BAC library construction involved electroporation to introduce the ligated DNA into *E. coli*. The transformation efficiency of the commercially available electrocompetent cells guarantees at least 10^{10} transformants/μg with the control plasmid (pUC19). Because of the high transformation efficiency of electrocompetent cells, the amount of size-selected DNA required to make a complete BAC library is less than that needed for the YAC library.

The vector used for chickpea BAC library construction was a binary vector, and hence it has the components necessary for A*grobacterium*-mediated transformation. Since chickpea is a dicotyledonous legume crop, and also that *A. tumefaciens* is capable of transferring 160 kb of the Ti plasmid DNA unidirectionally into a dicotyledonous plant, *Kalanchoe tubiflorea* (Miranda et al. 1992), we used the binary vector to construct a chickpea BAC library considering the feasibility of *Agrobacterium*-mediated transformation of large genomic fragments in chickpea.

Screening indicated that a negligible number of clones contained chloroplast DNA. The absence of an internal *Not*1 site was observed in the inserts in most of the 120 clones screened (Fig. 1). This indicated that the chickpea genome is not GC rich, as the *Not*1 enzyme is an octa-cutter and recognizes GC-rich regions. Empty clones were not observed in our library. The chickpea BAC library has 23,780 clones, representing 3.8-fold genome coverage, calculated from the estimated insert size and the genome size of chickpea.

This BAC library has approximately 95% probability of finding any chickpea genomic fragments based on the formula $N=\ln[1-P/\ln(1-I/GS)]$, where N = the number of clones; P = the probability of finding any genomic fragment in the library, I = average insert size and GS = genome size in Mbp.

Analysis of BAC clones identified using Ta96

Ta96 was shown to be linked to *Foc*3 at a genetic distance of 1 cM (Dev et al. 2003, manuscript in communication). This genetic distance equals an approximate physical distance of 360 kbp according to Winter et al. (2000). However, large variations were observed when genetic to physical distances were compared in other studies (Stein et al. 2000). This marker was mapped to linkage group 2 where other wilt R genes against races 1, 3, 4 and 5 of the same pathogen were located (Winter et al. 2000). There have been reports showing that resistance genes are clustered for different races of a pathogen in different plants, as well as in legumes (Kanazin et al. 1996; Yu et al. 1996). Screening of the BAC library with Ta96 identified two BAC clones of approximately 100 kb (15B1 and 7N13) (Fig. 2). The fingerprinting pattern revealed that these clones are not identical and have minimal overlapping (Fig. 3). The end sequences of these clones did not detect overlapping between them. To study their relationship, we screened the clones by PCR using primers designed from their end sequences (Table 1). Amplification of bands in both clones was observed when the primer designed from the left end sequences of 7N13 (7N13L) and the right end sequences of 15B1 (15B1R) were used (Fig. 4C, D). 7N13(R) did not amplify any band in 15B1 (Fig. 4B). Similarly, no band was amplified in 7N13 when 15B1 (L) was used (Fig. 4A). This amplification pattern indicated that these two clones overlapped at the interior sequences. The end sequences have been submitted to Genbank, and their accession numbers are AY356154, AY356155, AY356156 and AY356157. These end sequences are not composed of repetitive elements which can probably be attributed to higher gene content in this genomic region. This contradicts the earlier observation of finding many STMS markers, which amplify repetitive sequences surrounding the fusarium wilt resistance genes in that linkage group (Winter et al. 2000). However, this requires detailed investigation for confirmation.

Analysis of these two clones with eight RGA primers resulted in strong amplification of the bands. The bands were common between these two BAC clones and the parental DNA, although they are not polymorphic between the parents (data not shown). Upon the homology search, the right ends of 15B1 and 7N13 shared 89% sequence identity of 165 bp to a ribosomal protein of *Medicago truncatula* (mtgsp005e03), a closely related legume to chickpea and to a zinc finger-like protein motif of *Arabidopsis* (AT1G14580.1), respectively. Ribosomal proteins and zinc finger proteins are proven to play crucial roles in gene regulation. Since fusarium wilt R-genes show a recessive inheritance pattern (Mayer et al. 1997; Tullu et al. 1999), and their mechanisms are not yet known, our results provide only clues about the type of genes present in the genomic region that includes *Foc*3. Screening the library with other markers positioned near other R genes on linkage group 2 (Winter et al. 2000), and analyzing them, will help to generate information on the organization of R-genes in that particular linkage group 1 and also to generate tightly linked additional markers to various R genes and eventual R-gene isolation by positional cloning.

In summary, this is the first chickpea BAC library in a binary vector to be constructed. The library can be used to accelerate different aspects of genomic research in chickpea, including an analysis of the arrangement and development of microsatellites (Springer et al. 1994), studies of the structure and organization of multigene families and comparisons of specific regions of the genome with other closely related legume species. Cloning of large DNA fragments of genomic DNA in BAC vectors that may contain a gene cluster, is the first step towards gene isolation (Meksem et al. 2000).

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