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# Bacterial artificial chromosome (BAC) library resource for positional cloning of pest and disease resistance genes in cassava (*Manihot esculenta* Crantz)

J. Tomkins[1], M. Fregene [2,*], D. Main[1], H. Kim[3], R. Wing[3] and J. Tohme[2]
[1]*Clemson University Genomics Institute (CUGI), 304 BRC, Clemson, South Carolina, USA;* [2]*International Center for Tropical Agriculture, AA6713, Cali, Colombia, USA (*author for correspondence; e-mail M.Fregene@cgiar.org);* [3]*Arizona Genomics Institute, 303 Forbes Hall, Tuscon, Arizona, USA*

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**Abstract**

Pest and disease problems are important constraints of cassava production and host plant resistance is the most efficient method of combating them. Breeding for host plant resistance is considerably slowed down by the crop's biological constraints of a long growth cycle, high levels of heterozygosity and a large genetic load. More efficient methods such as gene cloning and transgenesis are required to deploy resistance genes. To facilitate the cloning of resistance genes, bacterial artificial chromosome (BAC) library resources have been developed for cassava. Two libraries were constructed from the cassava clones, TMS 30001, resistant to the cassava mosaic disease (CMD) and the cassava bacterial blight (CBB), and MECU72, resistant to cassava white fly. The TMS30001 library has 55 296 clones with an insert size range of 40–150 kb with an average of 80 kb, while the MECU72 library consists of 92 160 clones and an insert size range of 25–250 kb average of 93 kb. Based on a genome size of 772 Mb, the TMS30001 and MECU72 libraries have a 5 and 11.3 haploid genome equivalents and a 95% and 99% chance of finding any sequence, respectively. To demonstrate the potential of the libraries, the TMS30001 library was screened by southern hybridization using a cassava analog (CBB1) of the *Xa21* gene from rice that maps to a region containing a QTL for resistance to CBB as probe. Five BAC clones that hybridized to CBB1 were isolated and a *Hind III* fingerprint revealed 2–3 copies of the gene in individual BAC clones. A larger scale analysis of resistance gene analogs (RGAs) in cassava has also been conducted in order to understand the number and organization of RGAs. To scan for gene and repeat DNA content in the libraries, end-sequencing was performed on 2301 clones from the MECU72 library. A total of 1705 unique sequences were obtained with an average size of 715 bp. Database homology searches using BLAST revealed that 458 sequences had significant homology with known proteins and 321 with transposable elements. The use of the library in positional cloning of pest and disease resistance genes is discussed.

## Introduction

Disease and pest outbreaks occur frequently in major cassava growing regions with an attendant loss of food and income for resource poor farming communities and the erosion of valuable germplasm. An epidemic of the cassava mosaic disease (CMD), maybe the most devastating disease of cassava, during the late 1980s and early 1990s almost wiped out cassava production in Uganda. More than 70% of hectareage was destroyed before it was brought under control (Otim Nape *et al.*, 1997). The epidemic has since spread into Kenya, Tanzania, and the Democratic Republic of Congo, causing huge losses and hunger. Economic losses due to CMD in Africa have been estimated

at US$ 1.5 billion annually (Thresh *et al*., 1994). Cassava mosaic disease, caused by Gemini viruses of the genus Begomovirus (Family *Geminiviridae*) and transmitted by a white fly vector, *Bemisia tabaci* (Gennadius) is endemic in sub Saharan Africa, India and Sri Lanka, but is not known in the Americas. However since the early 1990s, a new biotype of *B. tabaci*, biotype B (also referred to as *B. argentifolia*), has been found feeding on cassava in the Americas (Polston and Anderson, 1997). This is a frightening prospect for cassava production in Latin America, considering that most of Latin American cassava germplasm is highly susceptible to CMD (Okogbenin *et al*., 1998).

Other white flies, including the species *Aleurotrachelus socialis* Bondar, *Trialeurodes variabilis* (Quaintance) *Aleurothrixus aepim* (Goldi), *Bemisia tuberculata*, are also a serious menace in their own right in the tropics. They cause major damage to cassava by feeding on the phloem of leaves leading to chlorosis, leaf fall, and an accompanying reduction in yield. White fly infestation of 1, 6 and 11 months after planting have resulted in yield losses of 5%, 42% and 72% (Vargas and Bellotti, 1981). Host plant resistance to CMD and white flies is the best option to combat these problems as they offer a low-cost, effective and long-term solution. A number of sources of durable resistance to CMD and white flies have been identified in cassava. The most widely deployed resistance to CMD was originally obtained from a wild relative of cassava, *M. glaziovii* and its inheritance is thought to be polygenic in nature (Nichols, 1947; Jennings, 1976). A high level of resistance controlled by a single dominant gene (*CMD2*) was recently identified in closely related Nigerian cassava land races (IITA, 1990; Akano, 2002). A large-scale screening of a cassava germplasm collection at the International Center for Tropical Agriculture (CIAT, its Spanish acronym) identified several cultivars with high levels of resistance to white flies. The cassava clone MECU72 had the highest level of nymphal mortality (72.5%).

Cassava's long growth cycle, heterozygous genetic background, and in around cassava gene pools via conventional breeding. A faster way would be the cloning the resistance genes and genetic transformation. The availability of reproducible transformation protocols (Chavariagga *et al*., 2002), a genetic map of cassava (Fregene *et al*., 1997; Mba *et al.,* 2001), and genetic mapping of resistance CMD and white flies (Akano *et al*., 2002; CIAT 2002) in cassava make this approach a feasible one. Resistance genes known only by their phenotypes, are best cloned by positional or map-based cloning. This requires the development of large-insert genomic libraries and ordering them into contigs that span the genome region carrying the gene(s) of interest. The bacterial artificial chromosome (BAC) system of cloning large DNA fragments is the preferred method for constructing large-insert libraries of genomes (Shizuya *et al*., 1992; Wang *et al*., 1995; Tomkins *et al*., 1999a, b). Apart from positional cloning, BAC libraries are a very important resource for end-sequencing of large insert clones, physical map development, and high-throughput sequencing of the genome. We describe here the construction and characterization of two BAC libraries from the cassava cultivars TMS30001 (CMD resistance) and MECU72 (White fly resistance).

## Materials and methods

### *Plant material*

The first BAC library was constructed from the cassava clones TMS30001 while a second one was later made from MECU72. Both libraries were constructed in a similar fashion. The TMS30001 library was developed at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, and it has the older, currently deployed source of resistance from *M. glaziovii*. Genetic mapping of this source of resistance has identified a region on linkage group D that explains 50% of phenotypic variance of CMD resistance (Fregene *et al*., 2000). The MECU72 clone is a cassava land race from Ecuador that is held in the cassava germplasm bank at CIAT, Cali, Colombia, and it shows the highest level of resistance to white flies. Genetic mapping of resistance is currently ongoing using a full-sib family of 280 plants obtained by crossing MECU72 to MCol2066 (CIAT 2002). A third BAC library is being constructed from another clone TME3 that has the new source of CMD resistance that is attributed to a single dominant gene designated *CMD2* (Akano *et al*., 2002). We describe here only construction of BAC libraries from TMS30001 and MECU72.

*BAC library construction*

BAC library construction was followed as described previously by Tomkins *et al.* (1999a, b) with the following modifications. Megabase size DNA was isolated from cassava clones TMS30001 or MECU72 and embedded in agarose plugs using a sucrose-based nuclei isolation buffer (Peterson *et al.*, 2000). High molecular weight genomic DNA in finely chopped plugs was partially digested with *Hin*d III (2 units per 100 $\mu$l of chopped plug material) for 20 min at 37 °C as described. Restriction enzyme digestion was stopped by placing on ice and addition of 1/10 volume 5 M EDTA. Partially digested DNA was size selected by two rounds of pulsed-field electrophoresis (CHEF MAPPER, Bio-Rad Corp). Conditions of pulsed field electrophoresis for the first round of selections was 1–40 s linear ramp, 6 V/cm, 120, 20 h run time, 1% agarose gels in 0.5X TBE. Gel sections from the 100 to 350 kb region, based on a 50 kb lambda molecular weight marker (NEB), were cut out and subjected to another round of size selection of 3–5 s linear ramp, 6 V/cm, 120, 20 h run time, 1% agarose gels in 0.5X TBE. Two gel slices, above 100 kb, were removed and the DNA isolated by electo-elution using the Bio-Rad Electro-eluter (Model 422) system.

The size-selected DNA fragments were ligated into the Hind III cloning site of pBeloBAC11 (Shizuya *et al.*, 1992) for the TMS30001 library and into pCUGI-1 for the MECU72 library using a vector:insert ratio of 1:8 and 30 ng of vector using 14 U of ligase in a final volume of 150 $\mu$l. Ligations were desalted and 2 $\mu$l were used to transform 20 $\mu$l of competent cells (DH10B, GIBCO BRL) by electroporation using a voltage of 320 V and resistance of 4Ω. Transformed cells were diluted with 1 ml of SOC (Sambrook *et al.*, 1989) and incubated at 37 °C for 60 min before being plated on selective medium (Luria–Bertani Medium) with 12.5 $\mu$g/$\mu$l of Chloramphenicol, 0.55 mM IPTG, and 80 $\mu$g/ml X-Gal. After a 20 h incubation at 37 °C the plates were placed in the dark at room temperature for another 20 h to permit stronger blue color development of non-recombinant colonies. White colonies were hand-picked and grown in 3 ml of liquid LB medium + 12.5 $\mu$g/$\mu$l Chloramphenicol for 14 h. Plasmid DNA was isolated by an automatic plasmid isolation robot (Kurabo Inc.), 1 $\mu$g of plasmid DNA was digested with 7.5 U of *Not* I, to liberate inserts, and separated by pulsed-field electrophoresis using a switch time of 5–15 s, 1% agarose, 0.5XTBE for 14 h to determine the sizes of inserts. Once the insert sizes were found to be adequate, an average of 80 kb or more, the rest of the ligation was utilized for additional transformations to obtain the desired number of clones for the library. The ligation was plated out as described above and recombinant white colonies were picked robotically (Q-bot robot, Genetix PLC) into 384-well microtiter plates (Genetic PLC) containing 50 $\mu$l freezing medium (Woo *et al.*, 1994). The microtitre plates were incubated overnight and then stored at −80 °C. Two copies of each library were made, a copy was sent to CIAT, while one was left at the Clemson University Genomics Institute (CUGI).

*BAC library characterization*

To estimate the distribution and average size of the clones, a total of 120 clones from the TMS30001 library and 370 clones from the MECU72 library were picked at random and grown overnight in 3 ml of liquid LB medium + 12.5 $\mu$g/$\mu$l Chloramphenicol. Plasmid DNA was isolated, digested with *Not* I restriction enzymes and inserts separated from the vector by pulsed-field electrophoresis as described above. Southern blots of the size-separated BAC inserts were prepared and hybridized with total genomic cassava DNA labeled with [$^{32}$P]dATP following standard methods (Sambrook *et al.*, 1989).

*BAC library screening*

To determine the representation of random low-copy number DNA clones, five low-copy number cassava genomic clones, GY 30, GY 42, CDY76, GY75, and a cassava analog, CBB1, of the rice bacterial blight resistance Xa-21 (Roland *et al.*, 1996, unpublished data) were used in screening the libraries. The map position, as RFLP markers, of these clones have been described elsewhere (Fregene *et al.*, 1997). A *Hind III* restriction enzyme fingerprint of the BAC clones that hybridized to CBB1 were probed using CBB1 as described by Marra *et al.* (1997). This was to understand the organization of this RGA in the cassava genome. High-density filters containing 18 432 clones, spotted twice, were prepared with the Q-bot robot

(Genetix Corp., Queensway, UK) using a $4 \times 4$ array on 22.5 $cm^2$ hybond N+ nylon filters (Amersham PLC.) and standard colony filters preparation methods (Sambrook *et al*., 1989). The filters were hybridized following methods established for cassava and described by Fregene *et al*. (1997). Screening for chloroplast DNA content was done according to Zhang *et al*. (1995).

*BAC-end sequencing*

Preparation of BAC DNA for end sequencing was done in a 96-well format using standard alkaline lysis miniprep techniques. Sequencing reactions were set up according to manufacturer's instructions for the Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA). Reactions were performed using forward and reverse universal primers. Samples were electrophoresed using 96-capillary ABI3700 automated sequencers. Base-calling was performed automatically using PHRED (Ewing and Green, 1998; Ewing *et al*., 1998), and vector sequences were removed by CROSS-MATCH (http://www.genome.washington.edu). High quality BAC end sequences (defined as those having >100 non-vector bases with a PHRED quality value >20) were used as queries in FASTX searches of the SWISS-PROT, Arabidopsis MIPS, Genbank NR, transposable element, and Genbank Plant cDNA databases. The BAC end sequences have been submitted to GenBank under accession numbers BH795110 to BH793356.

**Results and discussions**

*Library construction and characterization*

The BAC library from TMS30001 consists of a total of 55 296 clones in 144 384-well microtitre plates. This library was gridded onto 3 high density filters containing 18 432 clones per filter. About 4% of the clones do not contain inserts as judged from a random sampling of 120 clones from the library and insert size ranged from 20 to 150 kb with an average size of 80 kb. The MECU72 library had a total of 92 160 clones in 240 384-well microtitre plates. This library is gridded onto five high density filters. Based on a random sampling of 370 BAC clones from the library, the average insert size was 93 kb with a range of 25–250 kb, while no clones were found without insert. Figure 1 shows 42 randomly selected clones digested with *Not1*. The 370 randomly selected clones were grouped according to insert sizes, on an interval of 25 kb, and plotted against the number of clones in each insert size interval to determine the size distribution of the library (Figure 2). Based on this analysis, 48% of clones in this library are 100 kb or larger in size. A comparison between the cassava BAC libraries and libraries from other plant species held at the Clemson University Genomics Institute (CUGI) is shown in Table 1. Both libraries are publicly available and request for clones and colony filters can be made to the Clemson University Genome Institute (CUGI) by accessing their web page (http://www.genome.clemson.edu).

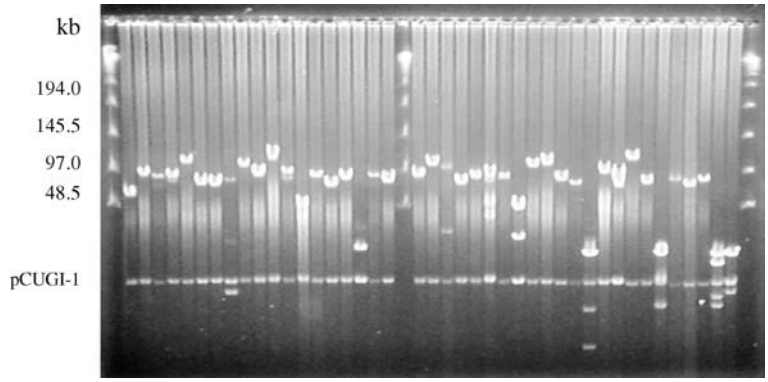


*Figure 1*. Random BAC clones from the MECU72 library digested with *Not1* and run in a 1% agarose gel in 0.5X TBE at 14 °C at 6 V/cm with a switch time of 5–15 s pulse time for 14 h.

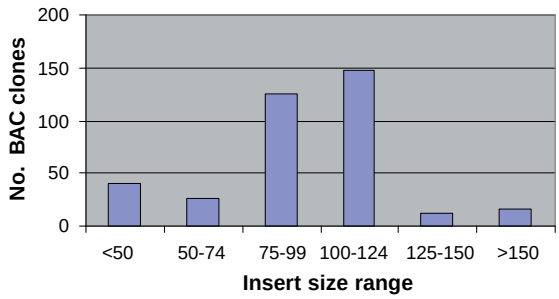


*Figure 2.* Size distribution of inserts from 370 BAC clones from the MECU72 library. Results reveal that over 48% of the inserts are 100 kb or larger and the average insert size is 93 kb.

Cassava has a diploid DNA content of 1.67 pg per cell nucleus (Awoleye *et al.*, 1994), corresponding to a haploid genome size of 772 Mb. Based on this, the coverage of the TMS30001 library is 5 genome equivalents while that of the MECU72 library is 11 genome equivalents. There are 95% and 99% probabilities of recovering any specific sequence in the TMS30001 and MECU72 libraries respectively. Southern hybridization of 5 low-copy number clones from cassava to high-density filters of the libraries had 5–12 hits per probing, which is close to the estimation of genome coverage given above. Results from screening both libraries with three different chloroplast genes revealed that $< 3\%$ of library sequences are chloroplast DNA (data not shown).

The BAC library was constructed with the objective of map-based cloning of disease and pest resistance genes in cassava. A good commencement point of cloning resistance genes is to identify BAC clones bearing resistance gene analogs (RGAs) already identified to be associated with regions of the genome with genes or QTLs for disease resistance. BAC clones that bear RGAs can be used to identify the number, organization and genome location of these genes. An example is CBB1, a *Xa-21* analog from cassava that maps to a region for a QTL that confers resistance to the cassava bacterial blight (CBB) (Jorge *et al.*, 2000). Figure 3 shows the Southern hybridization of clone CBB1, a *Xa-21* analog from cassava, and a *Hind III* restriction enzyme fingerprint of the five hybridizing BAC clones from the TMS30001 library using CBB1 as probe. Results reveal that the five clones that hybridized to the cassava *Xa21* analog appear to be three unique clones and the gene is present 2–5 times in individual BAC clones. Additional screening of the TMS30001 library with 12 RGAs of the NBS-LRR class of resistance genes isolated from cassava revealed a total of 43 BA clones that hybridized strongly to the RGAs (Lopez *et al.*, 2003, in preparation). Fingerprinting of the BAC clones enabled an assembly into 10 contigs and 19 singletons. One BAC clone was completely sequenced using a shotgun cloning strategy. Four partial resistance gene homologues

*Table 1.* A survey of some plant BAC libraries held at Clemson University Genomics Institute (CUGI), Clemson University, Clemson, South Carolina.

| Species | Number of clones | Average size (kb) | Genome coverage |
|---|---|---|---|
| Arabidopsis | 12 288 | 100 | 12X |
| Cassava (TMS30001) | 55 296 | 80 | 5X |
| Cassava (MECU72) | 92 160 | 93 | 11.3X |
| Common Bean | 30 000 | 90 | 4.3X |
| Maize | 13 2480 | 100 | 5.29X |
| Rice | 36 864 | 128 | 10.9x |
| Sorghum | 13 750 | 157 | 2.8X |
| Soybean | 73 728 | 130 | 8.6X |
| Sugarcane | 103 296 | 130 | 4.5X |
| Tomatoes | 129 000 | 117.5 | 15X |

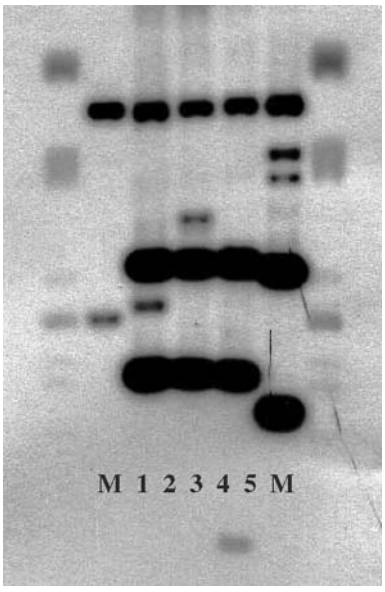


*Figure 3.* Southern hybridization of probe CBB1, a Xa2l analog from cassava, to *Hind III* restriction enzyme digests of five BAC clones. Lanes marked M ares Lambda/Pst1 molecular weight marker, lanes marked 1–5 are the individual BAC clones.

(RGH) and two complete RGHs, RGH1 and RGH2 contain an ORF of 3072 nucleotides without introns, were found (Lopez *et al*., 2003, in preparation).

*BAC-end sequencing*

BAC-end sequences, also known as sequence tagged connectors (STC), are a very important resource for scanning gene or repeat DNA content, data mining, chromosome walking, or as sequence based markers. A total of 2301 BAC clones were end-sequenced producing 1705 unique sequences with an average raw base count of 715 and an average HQ base count of 354. Database homology searches produced 198, 434, 452, 321, and 380 hits on the Swiss-Prot, Arabidopsis MIPS, Genbank NR, Transposable Element, and Plant cDNA databases, respectively. Results obtained are generally comparable to those found in other crop species such as grape, cotton, and maize (Tomkins *et al*., 2001a, b, 2002).

All database query results of the BAC-end sequencing in spread sheet format can be viewed and downloaded at the CUGI web-site: http://www.genome.clemson.edu/projects/stc/cassava/ME_MBa/. The sequences may also be downloaded via FTP at the same web site.

An attempt to anchor these BAC-end sequences to the cassava genetic map was made through a search for simple sequence repeat (SSR) motifs in the BAC ends. A total of 141 sequences were found with di-, tri-, or tetra-repeat motifs. These SSR markers are currently being mapped to the molecular genetic map of cassava.

Two large insert libraries have been constructed from the cassava clones TMS30001 and MECU72 with 5 and more than 11 genome equivalents. They are an important resource for map-based cloning of CMD, CBB, and white fly resistance genes. Current efforts are geared to developing physical maps, using BAC contigs, of regions of the genome that contain CMD and CBB resistance genes. Two approaches are currently followed, the 'candidate gene' approach and the fine-map approach.

The 'candidate gene' approach begins with RGA, isolated as PCR products using degenerate PCR primers from sequences of known plant resistance genes, or genes from differential expression studies that map to regions in the genome bearing QTLs or major genes controlling resistance. These genes are then used to isolate BAC clones that are fingerprinted and organized into contigs. The contigs would then be anchored to the genetic map of cassava by sequencing the ends of the BAC clones and mapping them, using a large mapping population. Based on the proximity of contigs to the resistance QTL or gene, unique homologs are picked out and used in genetic complementation experiments. The utility of the libraries for understanding the number and organization of candidate disease resistance genes was demonstrated in this study by the screening of the BAC library with a cassava analog of the rice bacterial blight resistance gene *Xa21*.

The fine map approach uses a large progeny mapping population and a modified bulked segregant analysis (Michelmore *et al*., 1991) to create a fine map of the region of the genome carrying the resistance gene of interest. Next, markers closest to the gene from the fine-map are used to isolate BAC clones that are fingerprinted and organized into contigs. The ends of the BAC clones are then sequenced and mapped as allele specific markers onto the fine-map. Two or three BAC clones that co-segregate with the resistance gene are then completely sequenced by shot-gun sequencing and the sequences searched for likely resistance genes for genetic complementation experiments.

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