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Construction and characterization of a bacterial artificial chromosome library of banana (*Musa acuminata* Colla)

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Abstract A bacterial artificial chromosome (BAC) library for banana was constructed from leaves of the wild diploid 'Calcutta 4' clone (*Musa acuminata* subsp. *Burmannicoides* 2n = 2*x* = 22). 'Calcutta 4' is widely used in breeding programs for its resistance to the current major disease of banana and is being used to build a genetic reference map of banana. As banana leaves are particularly rich in polyphenols and polysaccharides a protocol was adapted to isolate intact nuclei and high-molecular-weight (HMW) DNA. A total of 55,152 clones with an average insert size of 100 kb were picked. The frequency of BAC clones carrying inserts derived from chloroplast and mitochondrial DNA was estimated to be 1.5%. The coverage of the library is equivalent to 9.0-times the haploid genome. The BAC library was screened with 13 RFLP probes belonging to the 8 linkage groups of the consensus molecular map of banana. A total of 135 clones were identified giving an average of 10.38 clones for each locus. This BAC library will be a valuable starting tool for many of the goals of the recently emerged International *Musa* Genomic Consortium. One of our initial objectives will be to develop a banana physical map by BAC-FISH (fluorescent in situ hybridization) viewing the characterization of translocation break points.

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Introduction

Bananas are monocotyledons belonging to the *Musaceae* family. Banana cultivars are diploids, triploids or tetraploid's derived from natural hybridization between wild diploid *Musa* species. The main species involved are *Musa acuminata* Colla (A genome, 2n = 2*x* = 22) and *Musa balbisiana* Colla (B genome, 2n = 2*x* = 22). Bananas are important as an export crop, but they are first and foremost important as a crop for small holders and subsistence farmers in many developing countries in the tropical and subtropical regions of the world. Many different types of dessert and cooking bananas play a major role in food security (Frison and Sharrock 1999). Banana is a cheap and easily produced source of energy and, because of its high content in vitamins and minerals, it contributes significantly to the quality of the diet. Moreover, banana produces fruit all year round and grows in a wide range of environmental conditions, including those less favorable for the production of other staple foods (Frison and Sharrock 1999). However, yields of banana remain low and cultivars are susceptible to an ever-increasing range of fungal, viral and insect diseases.

For many decades, banana breeding was unsuccessful, in particular because of polyploidy, sterility and parthenocarpy; but in the past 10 years *Musa* researchers world-wide have made a number of important breakthroughs, and now the first high yielding, disease-resistant varieties are available for wide-scale testing and distribution to farmers (Silva et al. 2000; Bakry et al. 2001). Nevertheless, it is clear that a significant banana research effort is still needed to address threats to production.

The *Musa* genomes display several characteristics that could be exploited for gaining a fundamental insight into the genomes of other species. The small size of the haploid genome (around 600 Mb, Afza et al. 1993; Dolezel et al. 1994), the different ploidy levels and coex-

istence of various genome combinations in the existing crops, as well as the combination of parthenocarpy with sterility and vegetative propagation, place the banana as a good model to study gene expression in different chromosomal environments. To reinforce banana genomic research a "*Musa* Genomics Consortium" has recently been established (http://www.inibap.org/research/promusa.eng.htm)

One of the first tools necessary to develop banana genomics research is a banana BAC library (Bacterial Artificial Chromosome) (Shizuya et al. 1992). The BAC cloning system has become an invaluable tool because of its ability to stably maintain large DNA fragments and its ease of manipulation (Shizuya et al. 1992; Woo et al. 1994; Wang et al. 1995). BAC libraries have been established for all major crops and are now being developed for less investigated species such as melon (Luo et al. 2001), papaya (Ming et al. 2001) and peach (Wang et al. 2001).

The accession chosen to construct the BAC library is 'Calcutta 4', (*M. acuminata* subsp. *Burmannicoides*, De Langhe), a wild diploid (AA) present in most collections and widely used in breeding program for its resistance to black leaf streak (*Mycosphaerella fijiensis*) and yellow sigatoka (*Mycophaerella musicola*), two of the current major diseases of banana. 'Calcutta 4' is also resistant to Panama disease (*Fusarium oxysporun* f. *cubense)* and to nematodes. This clone is also being used to build a genetic reference map of banana.

In this paper we describe the construction and characterization of a *M. acuminata* 'Calcutta 4' BAC library including the determination of insert sizes and chloroplast and mitochondria DNA contamination. The difficulties encountered are discussed. This BAC library will be a valuable starting resource for many goals of the *Musa* Genome Consortium. One of our first objectives is to use the BAC library to develop a banana physical map by BAC-FISH hybridization (Fluorescent in situ hybridization), viewing the identification of translocation break points.

Materials and methods

Plant material

The accession 'Calcutta 4' (2n = 2*x* = 22) comes from the CIRAD germplasm collection located in Guadeloupe (French West Indies). The plants were transferred and maintained in a greenhouse at the CIRAD campus of Montpellier (France) where the young leaves were harvested.

High-molecular-weight (HMW) DNA isolation

Nuclei were isolated from leaves according to Zhang et al. (1995) (http://www.genome.clemson.edu/protocols) with few modifications. Briefly, prior to homogenization, tissues were treated with ether to wash off waxes and make nuclei more friable. Young leaves were ground in liquid nitrogen and nuclei were isolated, rinsed several times in washing buffer plus 1% of Polyvinylpyrrolidone (PVP 40). PVP 40 was added to the extraction washing-buffer to reduce the content of polyphenolic substances in the banana-leaf homogenate. Between the washing steps the leaf homogenate was filtered through nylon filters (250, 100 and 40 µm) to reduce cell debris contamination (Faure 1993). Centrifugation at low speeds (57 g/2 min), as well as centrifugation through Percoll gradient (37.5%) (Peterson et al. 2000), were also employed. Finally, the nuclei were resuspended in 1 ml of filtered extraction buffer without β-mercaptoethanol and PVP, and embedded in 1.0% low-melting-point agarose plugs (In Cert Agarose, BMA, Rockland, USA). Agarose plugs were incubated for 24 h in lysis buffer, and stored in TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 8.0) at 4 °C. All buffers were prepared according to Zhang et al. (1995). HMW DNA integrity was tested by pulsed-field gel electrophoresis (PFGE) using a CHEF MAPPER apparatus (Bio-Rad, U.K.) at 9 V/cm, with a 3–5 s pulse, for 3:30 h at 14 °C in 0.5 × TBE buffer (0.09 M Tris–borate, 0.09 M boric acid, 0.002 M EDTA).

BAC library construction

Agarose plugs were chopped into small pieces with a glass blade and stored in TEX (10:1 TE with 0.01% of Triton X-100). Chopped plugs were incubated in 1×*Hin*dIII restriction buffer (Gibco BRL, USA) with 4 mM spermidine for 30 min on ice. Ten units of *Hin*dIII was added to the chopped plugs and allowed to diffuse for 30 min on ice. For partial digestions, reactions were incubated for 2 min and 30 sec at 37 °C, and then stopped by adding one-tenth of the total volume of 0.5 M EDTA, pH 8.0. Partially digested DNA was size-selected by PFGE. Two types of migration conditions were tested. For the first type (one size-selection), DNA was analysed in 1% agarose gels in three steps at 14 °C in 0.5 × TBE buffer: (1) 6 V/cm for 1 h with 90 s of switch time and an angle of 60°, (2) 18 h with 2 s of switch time and an angle of 60°, and (3) 10 h with 20 to 50 s of switch time and an angle of 60°. For the second type (two size-selection), DNA was separated in 1% agarose gels at 6 V/cm at 14 °C in 0.5 × TBE for 20 h with a 15 to 25 s of switch time and an angle of 120°. The region of the gel containing DNA between 125 kb and 350 kb was cut. The agarose slice was loaded onto a new 1% agarose gel. The migration was then performed at 6 V/cm, with 3 s of switch time, and an angle of 60°, for 20 h. The region of the gel containing the HMW DNA was cut and the DNA was electro-eluted from the agarose block following the method described by Strong et al. (1997) but using 100 µl of 1 × TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA). After the electro-elution, DNA concentration was estimated in agarose gels using a dilution series of lambda DNA as a concentration standard. The commercial vector "pIndigoBAC-5 *Hin*dIII–Cloning Ready" from Epicentre, USA, was used. Four ligations were performed in 100-µl reactions containing 33 ng (10 vectors/1 insert), 66 ng (5 vectors/1 insert) and 333 ng (1 vector/1 insert) of partially digested DNA, 25 ng of linearized and dephosphorylated pIndigoBAC-5 *Hin*dIII–Cloning Ready, 1 × ligase buffer, 1 mM ATP and 4.5 units of T4 DNA ligase (Promega, USA) at 16 °C overnight. After ligation, the solutions were desalted. One microlitre of the ligation was used to electroporate 19 µl of *Escherichia coli* ElectroMAX DH10B cells (BRL) using a BRL Cell-Porator System according the manufacturers' recommendations, but reducing the voltage of transformation (charge rate) from 400 to 350 V. After transformation, the cells were resuspended in 1,000 µl of SOC medium (2% Bacto tryptone, 0.5% Bacto Yeast extract, 10 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0) and incubated for 1 h at 37 °C with shaking at 225 rpm. The SOC medium with the cells was plated on LB plates containing 12.5 µg/ml of chloramphenicol, 50 µg/ml of X-Gal and 25 µg/ml of IPTG (isopropyl–thiogalactoside), and incubated at 37 °C over-night. White recombinant colonies were picked using a Flexis picker (Genomic Solutions, U.K.) and transferred to 384-well plates containing 50 µl of LB freezing buffer (Woo et al. 1994). The plates were incubated overnight at 37 °C, duplicated and stored at –80 °C.

Table 1 Banana BAC library filter-hybridization results in using eight single-copy and five duplicated (*) banana RFLP probes anchored to eight different linkage groups (LG) of the consensus banana genetic map (http://www.cirad.fr/presentation/programmes/biotrop/resultats/biositecirad/banana.htm)

Probes	LG	No. hits	Addresses
pMaCIR 309*	1	05	35A-08, 83B-03, 84B-21, 102A-08, 137N-04
pMaCIR 508*	2	30	03G-16, 05H-17, 14F-12, 19B-06, 50D-15, 60I-09, 61D-17, 69E-16, 70C-10, 80L-10, 83E-03, 84E-13, 97P-01, 98B-07, 108K-06, 108M-08, 110D-12, 116O-12, 118F-04, 119H-11, 119P-22, 121J-24, 121P-08, 126G-20, 129I-22, 130E-03, 130E-23, 131L-12, 134P-24, 139C-13, 134P-24, 139-C13
pMaCIR 613	2	10	3P-12, 19G-13, 41D-21, 61O-24, 95E-04, 105A-08, 125J-11, 136C-10, 139J-06, 140H-12
pMaCIR 257*	3	06	68P-03, 82I-11, 93B-08, 93M-21, 130K-24, 143H-04
pMaCIR 1111*	3	04	15I-07, 37A-21, 37P-23, 114M-04
pMaCIR 36	4	20	7E-23, 15O-13, 17B-21, 46G-23, 50I-24, 59I-20, 62A-05, 62L-04, 74J-14, 77O-13, 87G-21, 95L-01, 107F-09, 109B-21, 113D-18, 115O-18, 128P-20, 129C-02, 134I-07, 142N-04
pMaCIR 614	4	04	48D-18, 64G-22, 103 J-03, 110N-18
pMaCIR 209	5	12	9G-10, 22D-22, 26C-10, 29A-15, 29P-23, 31N-20, 50B-17, 65M-07, 104O-20, 114J-24, 130N-21, 139B-10
pMaCIR 1006*	5	13	17O-15, 22D-23, 40K-03, 79A-06, 81I-01, 84H-11, 91F-21, 104L-21, 106I-20, 124I-14, 133F-12, 135K-07, 138H-06
pMaCIR 560	6	21	01-P13, 03P-12, 06B-16, 09L-09, 11D-17, 19C-07, 19G-13, 34F-11, 54N-07, 57K-13, 63I-14, 69F-05, 85B-18, 94O-19, 96J-23, 119L-23, 127A-21, 130K-08, 136C-08, 136H-19, 144G-12
pMaCIR 231	8	13	19C-19, 30A-05, 31D-08, 33A-08, 57B-24, 81K-07, 103N-17, 105M-05, 107N-22, 108L-22, 115A-06, 124B-16, 143C-22
pMaCIR 715	8	09	19A-08, 36K-23, 40B-10, 55D-14, 59P-13, 84B-21, 90K-06, 130I-12, 130K-15
pMaCIR 112	9	17	15G-08, 29M-05, 42M-22, 65D-01, 78P-23, 83D-15, 84F-10, 89P-23, 93E-20, 106B-07, 120M-13, 126D-01, 132J-02, 137C-03, 139D-13, 139F-07, 143N-13

BAC library screening

High-density filters were made using a Flexys robot. Each high-density filter contains 18,432 double-spotted clones to avoid false positives. Hybridization and washes were performed as described earlier (http://www.genome.clemson.edu/protocols). Filters were exposed for 24–72 h using Fuji Medical X-Film (Super RX-100 NIF). Probes used for library screening are listed in Table 1.

BAC DNA isolation

Individual BAC clones were inoculated in 3-ml overnight-LB cultures with 12.5 µg/ml of chloramphenicol. Supercoiled BAC DNA was isolated using a QIAGEN BIO ROBOT 9600 (Qiagen GmbH, Germany). BAC DNAs were digested with *Not*I to release the BAC insert. The digested clones were separated by PFGE 6 V, a switch time from 5 to 15 s, an angle of 120 °and run of for 5 h.

Results

The main problem we encountered during banana HMW extraction was the presence of high levels of polyphenols and carbohydrates. Different methods of DNA extraction were tested (Faure 1993; Zhang et al. 1995; Peterson et al. 2000). The best results were obtained with a combination of them, including steps of filtration through nylon filters (250, 100 and 40 µm), low-speed centrifugation and centrifugation through a Percoll gradient. These steps allowed to eliminate cell debris, intact cells and starch grains that typically pellet with nuclei. The main difficulty was to find the best compromise between DNA concentration and DNA quality. An excessive DNA purification reduces too much of the DNA concentration at the end of the extraction. On the other hand an excess of cell impurities reduces the DNA quality. In both situations a high number of false positives (white colonies without inserts) were obtained after cloning. After the nuclei were embedded in agarose plugs and lysed, plug color changed depending on the contamination level. DNA concentration was high in dark-brown plugs and low in light-yellow plugs. The DNA quality was inversely proportional. It is interesting to note that the digestibility of DNA from dark-brown plugs is not affected. The problem is identified after transformation and thus probably occurs during ligation. Using color as a parameter the best results were obtained with dark-yellow plugs that represent a good compromise between DNA quantity and quality.

Two methods of size selection were tested. When one cycle of size-selection DNA was used the size average was 100 kb, while when two cycles of size-selection was used the size average was enhanced to 120 kb. The library was established from two ligations having a 5 vector × 1 insert ratio (mainly from one involving an insert selected through one size-selection), and consists of 55,152 clones stored in 144-well 384 microtiter plates. The insert size of the BAC library was estimated from 130 randomly selected clones and ranged from 40 to 330 kb, with an average of 100 kb (Fig. 1). The majority of the insert sizes (75%) fall into the range of 75 to 175 kb (Fig. 2). The number of *Not*I sites ranged from 0 to 3.

To examine the stability of banana BAC clones in *E. coli*, we analyzed the *Not*I restriction patterns of six large BAC clones in the 0 and 100 generations. No visible change was found (data not shown).

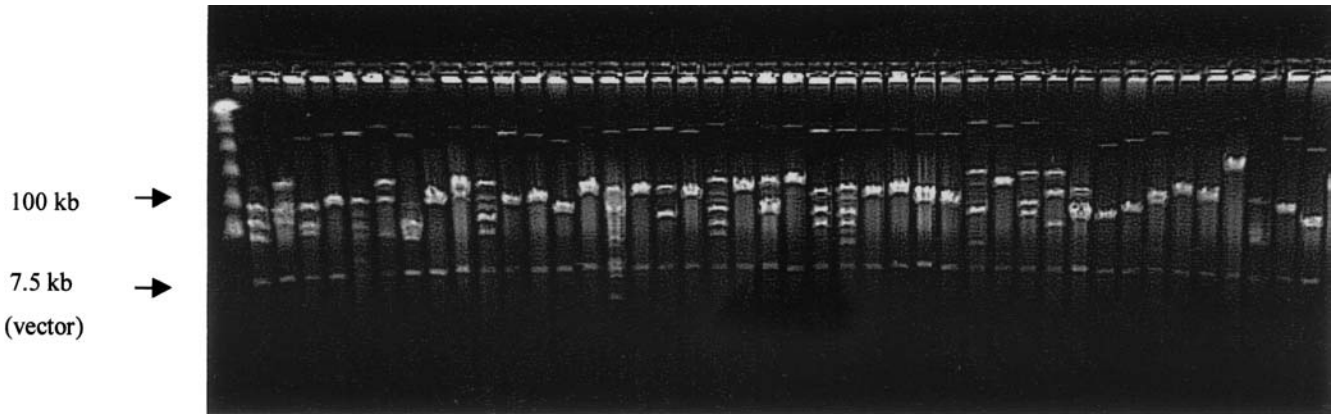

Fig. 1 Forty two random BAC clones from the banana BAC library digested with *Not*I and separated by PGEF (5–15 s and 6 V/cm at 14 °C in 0.5 × TBE buffer for 5 h). *Line 1:* lambda ladder PFG marker (New England Biolabs)

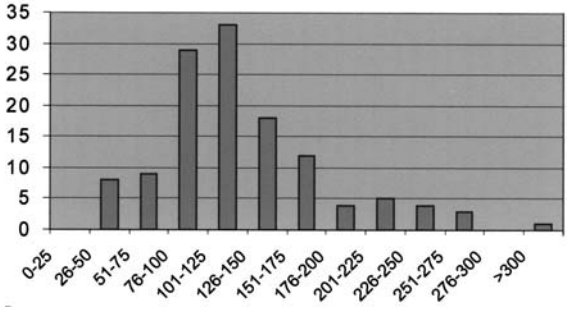

Fig. 2 Insert size distribution of 130 clones randomly picked from the Banana BAC library

To estimate the representation of organellar DNA, high-density colony filters were screened with a spinach chloroplast gene, the large Rubisco subunit (1.5 kb) and two wheat mitochondrial genes, Cox 1 (1.3 kb) and Cox 2 (0.6 kb) as probes. The contamination of the BAC library with organellar DNA was estimated to be 1.5% (887 clones) for chloroplast DNA and 0.067% (37 clones) for the mitochondrial DNA.

The size of the banana haploid genome is estimated to 600 mpb (Afza et al. 1993; Dolezel et al. 1994). After subtracting the number of organellar DNA clones, we estimated the coverage of the haploid genome to be 9.0 x. To corroborate this estimation, the BAC library was screened with 13 banana genetic markers (RFLP probes) linked to eight linkage groups of the consensus molecular map (http://www.cirad.fr/presentation/programmes/biotrop/resultats/biositecirad/ banana.htm). A total of 135 BAC clones was identified resulting in an average of 10.38 BACs/probe (Table 1).

Discussion

BAC library construction can be a time-consuming challenge. Good preparations of vector and size-selected HMW DNA inserts share in the success of BAC library construction. Most of the time is spent on repeatedly optimizing their preparation (Luo et al. 2001). We used a commercial vector to avoid the first possible difficulties. The predominant problems involved in trying to isolate HMW plant nuclear are to break plant cell walls without damaging nuclei, to separate chloroplasts from nuclei (since copies of the chloroplast genome may comprise the majority of total DNA within a plant cell), prevent secondary compounds such as polyphenols to interact with the nuclear DNA, and avoid that carbohydrate matrices trap nuclei (Katterman and Shattuck 1983; Guillemaut and Maréchal-Drouard 1992; Peterson et al. 2000). In banana leaves the presence of polyphenols and carbohydrates is particularity important (Gooding et al. 2001). Indeed their high level was the major problem we encountered. Various steps were added to standard protocols to obtain high quality HMW DNA. However these steps reduced the yield of nuclear HMW DNA in the plugs. Therefore a compromise had to be established between the quality and quantity of HMW DNA.

To increase the average insert size we tested two different protocols. One size-selection in a multi-state run (29 hours at total) and two cycles of size selection. As observed by other researchers, two cycles of size-selection had lower transformation efficiency, but enhanced the average insert size (Woo et al. 1994; Lin et al. 1999). During ligation and transformation procedures it was observed that the same degree of efficiency (the number of transformed cells per µl of ligation) was found with different ratios of insert to vector (10 vectors × 1 insert, 5 × 1 and 1 × 1). Previous work also mention different ratios for BAC library construction (Lin et al. 1999; Song et al. 2000; Luo et al. 2001; Wang et al. 2001). The contamination-degree of organelle DNA found was 1.5% and 0.067% for chloroplast and mitochondrial DNA respectively. This result is comparable with other BAC libraries constructed from plant leaves.

The BAC library is estimated to represent nine-times the haploid genome. The genome is thus well represented and the library will be suitable for many applications.

In particular, this BAC library will be a valuable tool for many of the goals of the recently emerged International *Musa* genomic consortium, such as gene cloning, genome sequencing, physical mapping and comparative genomics in monocots.

Our first objective is to use the BAC library to characterize translocations in the banana genome. Indeed, translocations are frequently observed at meiosis in *M. acuminata* (Shepherd 1999), and their poor knowledge makes it difficult to understand the inheritance of desirable characters and to steer their transfer to progeny. In banana, in situ hybridization was used to study the genome structure of cultivars (Osuji et al. 1997, D'Hont et al. 2000) and for localizing repeated sequences (Dolezelova et al. 1998; Osuji et al. 1998; Valarik et al. 2002). BAC inserts used as a probe in FISH (Fluorescent in situ hybridization) offer a great potential to relate the genetic map to chromosomes (Jiang et al. 1995; Peterson et al. 1999; Jackson et al. 2000). We are thus intending to use the BAC library to develop a physical map of banana and characterize the translocations of the banana genomes.

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