Construction of bacterial artificial chromosome (BAC) libraries of banana (*Musa acuminata* and *Musa balbisiana*)

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Abstract — Introduction. This protocol applies to the characterization of genome structure and evolution of *M. acuminata* and *M. balbisiana* genomes; construction of physical maps around markers of interest and QTLs; comparative genomic studies with other monocot species; and study of banana streak virus (BSV) integration patterns in banana nuclear genomes. The principle, key advantages, starting plant material, time required and expected results are presented. **Materials and methods**. This part describes the required materials, and the protocols for isolation and lysis of banana nuclei; high-molecular-weight (HMW) DNA *Hind* III digestion and pulsed-field gel electrophoresis (PFGE) analysis; large-scale HMW DNA *Hind* III digestion and ligation to the pINDIGOBAC–5 vector; and analysis of recombinant BAC clones. It mentions the main problems which can occur. **Results**. The described protocols will enable the efficient construction of BAC libraries from *Musa* species. These libraries can be used to construct physical maps and select specific genomic regions to be sequenced.

France / *Musa* sp. / methods / electrophoresis / DNA / genetic markers / genetic maps

Construction de bibliothèques de chromosomes artificiels de bactérie (BAC) de bananier (*Musa acuminata* et *Musa balbisiana*).

Résumé — Introduction. Le protocole s'applique à la caractérisation de la structure du génome et à l'évolution des génomes de *M. acuminata* et de *M. balbisiana* ; à la construction de cartes physiques autour des marqueurs d'intérêt et des QTLs ; aux études de génomique comparative avec d'autre espèce de monocotylédones ; à l'étude des modèles d'intégration du virus BSV dans les génomes nucléaires de la banane. Le principe, les principaux avantages, le matériel végétal utilisé, le temps nécessaire et les résultats attendus sont présentés. **Matériel et méthodes**. Cette partie décrit le matériel de laboratoire nécessaire, et les protocoles permettant l'isolement et la lyse des noyaux de cellules de bananier ; la digestion *Hind* III de ADN à haut poids moléculaire (HPM) et l'analyse électrophorèse sur gels à champ pulsé ; la digestion *Hind* III à grande échelle de ADN à HPM et ligation au vecteur pINDIGO-BAC-5 ; l'analyse des clones recombinants de BAC. Elle mentionne les principaux problèmes pouvant se poser. **Résultats**. Les protocoles présentés permettent de construire des bibliothèques de BAC à partir d'espèces du genre *Musa*. Ces bibliothèques peuvent être utilisées pour construire des cartes physiques et sélectionner des régions du génome à séquencer.

France / *Musa* sp. / méthode / électrophorèse / ADN / marqueur génétique / carte génétique

Fruits, 2008, vol. 63, p. 375–379

DOI: 10.1051/fruits:2008037
www.fruits-journal.org

1. Introduction

Application

The protocol applies to:
– characterization of genome structure and evolution of *Musa acuminata* and *Musa balbisiana* genomes,
– construction of physical maps around markers of interest and QTLs,
– comparative genomic studies with other monocot species (*e.g.*, rice, sorghum, oil palm) [1],
– study of banana streak virus (BSV) integration patterns in banana nuclear genomes [2],

– sequencing of selected regions of the *Musa* genome [3, 4] and systematic sequencing of nuclear, mitochondrial and chloroplast genomes.

Principle

Banana nuclei are isolated from leaf tissues using a modification of the protocol developed by Zhang *et al.* [5] to eliminate high levels of polyphenols [6] and polysaccharide contaminants or using a flow sorting approach [7]. High-molecular-weight (HMW) DNA is then released from the nuclei by mild lysis and partially digested with the restriction enzyme *Hind* III. Pulsed-field gel electrophoresis is employed to separate banana HMW genomic DNA and the region covering fragments between (100 and 300) kbp is then dissected out. Then, by electroelution [8], the high molecular genomic DNA (100–300 kbp) is recovered and ligated to the *Hind* III dephoshorylated BAC vector pIndigoBAC–5. The size of the resulting recombinant bacterial artificial chromosomes (BACs) containing banana genomic DNA fragments is visualized by NotI digestion followed by pulsed-field gel electrophoresis analysis. This protocol was successfully employed to construct BAC libraries from both *Musa acuminata* diploid [1, 9] and *Musa balbisiana* [1, 7].

Key advantages

(a) The method was successfully used for constructing BAC libraries of both *Musa acuminata* and *Musa balbisiana* genomes and can be employed to construct BAC libraries from other *Musa* wild and cultivated genotypes (*e.g.*, Cavendish).

(b) The nuclei isolation protocol here described can be used as a basis for other technologies such as Fiber-FISH (Fluorescence *in situ* Hybridization) and flow cytometry [7].

(c) This method enables highly efficient cloning of large fragments (up to 300 kb) of banana nuclear genomic DNA. The BAC libraries from *Musa acuminata* and *Musa balbisiana* were successfully used for comparative genomics approaches with rice [1], characterizing BSV integration patterns [2], and investigating nuclear genome structure [3, 4].

Starting material

Approximately 30 g of fresh and healthy fully opened banana leaves are necessary.

Time required

Starting from banana leaf material, 9 days are required to complete the BAC library preparation: day 1, isolation of nuclei, lysis of nuclei; day 2, test digestion conditions with *Hind* III, pulsed-field gel electrophoresis; day 3, large-scale *Hind* III digestion, pulsed-field gel electrophoresis first step; day 4, pulsed-field gel electrophoresis second step; day 5, electroelution of HMW DNA, ligation to pINDIGOBAC–5 vector; day 6, transformation of *E. coli* with ligation mix, plating of recombinant bacteria; day 7, pick recombinant BAC colonies, inoculation of BAC clones in 2YT liquid medium; day 8, purification of BAC DNA, digestion with NotI; day 9, pulsed-field gel electrophoresis analysis of recombinant BAC clones.

2. Materials and methods

2.1. Laboratory materials

The protocol requires:
– pulsed-field gel electrophoresis apparatus,
– centrifuge and microcentrifuge,

– T4 DNA ligase with buffer,
– NotI, *Hind* III restriction enzymes with specific buffers (Gibco-BRL),
– proteinase K,
– cell electroporator,
– pipetting material,
– agarose gel electrophoresis material,
– lambda ladder PFG marker (New England Biolabs),
– agarose plug 80 µL molds (BioRad),
– InCert agarose (BMA),
– pINDIGOBAC–5 Hind III–Cloning Ready vector (Epicentre, USA),
– Gold SeaKem agarose (BMA),
– diethylether.

2.2. Protocols

Isolation and lysis of banana nuclei

• Step 1
Grind the leaf material in liquid nitrogen and incubate the cell extracts at 4 °C for 20 min in modified HB 1× buffer (10 mM Tris, 10 mM EDTA, 80 mM KCl, 20 mM NaCl, 1 mM spermine, 1 mM spermidine, 45 mM β-mercaptoethanol, 0.5% Triton X–100, 0.5 M sucrose, 1% of polyvinylpyrrolidone (PVP–40), 0.25% diethylether).

Note: PVP–40 and diethylether are added to the HB extraction buffer to reduce the oxydation of polyphenolic compounds present at high levels in the banana leaf homogenate.

• Step 2
Filter leaf homogenate through a series of nylon filters [(250, 100 and 40) µm pore diameter]. Centrifuge the cell extract at 57 *g* for 2 min.

Note: these two steps enable separation of nuclei from cellular debris (cell wall fragments) and intact organelles (chloroplast, mitochondria).

• Step 3
Pellet the isolated nuclei by centrifugation at 850 *g* for 5 min and resuspend the nuclei in 1 mL filtered HB 1× extraction buffer without β-mercaptoethanol and PVP–40.

• Step 4
Embed the banana nuclei in 1.2% low-melting-point agarose plugs (InCert Agarose, BMA, Rockland, USA) by mixing an equal volume of nuclei suspension and liquefied agarose and pouring the nuclei-agarose mixture into 80–µL plug molds (Bio Rad) to form agarose plugs.

• Step 5
Incubate the agarose plugs for 16 h at 42 °C in 50 mL of Nuclei Lysis buffer (0.5 M EDTA, 1% lauroylsarcosine, 1 $mg \cdot mL^{-1}$ proteinase K, 45 mM β-mercaptoethanol).

• Step 6
Inactivate residual proteinase K by incubating the agarose plugs in 50 mL of 500 mM EDTA + 0.1 M phenylmethyl sulfonyl fluoride (PMSF) for 1 h at room temperature.

• Step 7
Rinse the agarose plugs in ET buffer 10 mM Tris + 50 mM EDTA, pH 8.0, for at least 1 h, then in TE [10:10] for an additional 2 h and, finally, for 1 h in TE [10:1] (10 mM Tris + 1 mM EDTA pH 8.0).

High-molecular-weight (HMW) DNA *Hind* III digestion and pulsed-field gel electrophoresis (PFGE) analysis

• Step 8
Chop the agarose plug using a sterile razor blade into small squares of approx. 0.1 cm and incubate the chopped plugs (1 mL per agarose plug) in 1× *Hi*nd III restriction buffer (Gibco BRL, USA) with 4 mM spermidine for 30 min on ice.

• Step 9
For partial digestions, add 10, 20, 30, 40 and 50 units of *Hi*nd III restriction enzyme to each chopped plug and allow the restriction enzyme to diffuse for 30 min on ice.

• Step 10
Incubate the *Hind* III digestion reactions for 15 min at 37 °C, then stop the activity of the *Hind* III restriction enzyme by adding one-tenth of the total digestion volume of 0.5 M EDTA, pH 8.0.

• Step 11
Migrate the *Hind* III digested agarose plugs in 1% Gold SeaKem agarose (BMA) by pulsed-field gel electrophoresis (PFGE) using the following conditions: 6 $V \cdot cm^{-1}$, 1–50-sec pulse, 16 h, angle 120°, 14 °C, using 0.5 × TBE buffer (0.09 M Tris-borate, 0.09 M boric acid, 0.002 M EDTA).

Large-scale HMW DNA *Hind* III digestion and ligation to the pINDIGOBAC–5 vector

• Step 12
Once the most appropriate concentration of *Hind* III restriction enzyme is determined, repeat preparation of agarose plugs for *Hind* III digestion, as described in steps 8 to 10, using 10 agarose plugs.

• Step 13
Migrate the *Hind* III-digested agarose plugs in 1% Gold SeaKem agarose (BMA) by pulsed-field gel electrophoresis (PFGE) using the following conditions: 6 $V \cdot cm^{-1}$, 1–50-sec pulse, 16 h, angle 120°, 14 °C, using 0.5 × TBE buffer (0.09 M Tris–borate, 0.09 M boric acid, 0.002 M EDTA).

• Step 14
Following electrophoresis, excise the edges of the gel containing the marker (Lambda ladder PFG marker, New England Biolabs) and a small portion of digested high-molecular-weight banana DNA and stain them with ethidium bromide. Mark the area between (100 and 300) kb.

• Step 15
Excise two portions of the gel containing banana HMW DNA ranging from (100 to 175) kb and (175 to 300) kb, respectively.

• Step 16
Migrate the two gel portions for a second time in 0.9% Gold SeaKem agarose (BMA) by pulsed-field gel electrophoresis (PFGE) using the following conditions: 6 $V \cdot cm^{-1}$, 3-sec pulse, 16 h, angle 120°, 14 °C, using 0.5 × TBE buffer (0.09 M Tris–borate, 0.09 M boric acid, 0.002 M EDTA).

• Step 17. Cut the region of the gel containing the HMW banana DNA with a size above 100 kb.

• Step 18
Electro-elute the HMW banana DNA from the agarose block as described by Strong *et al.* [8] using 100 µL 1× TAE buffer (0.004 M Tris-acetate, 0.001 M EDTA).

• Step 19
Recover the purified HMW DNA and estimate the concentration of the recovered banana HMW DNA in agarose gel (1% in TAE 1×) using dilution series of lambda DNA as a DNA concentration standard.

• Step 20
Ligate 200 ng of purified HMW banana DNA to 25 ng of pINDIGOBAC-5 *Hind* III–Cloning Ready vector (Epicentre, USA) using T4 DNA ligase (Gibco-BRL) in a final volume of 100 µL.

Analysis of recombinant BAC clones

• Step 21
Use 1 µL of the ligation to electroporate 20 µL of *Escherichia coli* ElectroMAX DH10B cells (BRL), using a BRL Cell-Porator System according to the manufacturers' recommendations, but reducing the voltage of transformation (charge rate) from (400 to 330) V.

• Step 22
Following electroporation, resuspend the DH10B bacterial cells in 1 mL of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose, pH 7.0) and incubate for 1 h at 37 °C with shaking at 225 rpm.

• Step 23
Plate an aliquot of 50 µL of the resuspended bacterial cells on 2YT plates containing 12.5 $\mu g \cdot mL^{-1}$ chloramphenicol, 50 $\mu g \cdot mL^{-1}$ X-Gal and 25 $\mu g \cdot mL^{-1}$ IPTG (isopropyl–thiogalactoside), and incubate at 37 °C overnight.

• Step 24
Incubate white recombinant colonies in 10 mL of 2YT medium with 12.5 $\mu g \cdot mL^{-1}$ chloramphenicol for 16 h at 37 °C and isolate BAC DNA using the BAC isolation kit (Qiagen).

• Step 25
Digest 500 ng of purified BAC DNA with NotI overnight to release the BAC insert.

• Step 26
Separate the NotI-digested BAC DNA in 1% Gold SeaKem agarose (BMA) by pulsed-field gel electrophoresis (PFGE) using the following conditions: 9 $V \cdot cm^{-1}$, 5–15-sec pulse, 5 h, angle 120°, 14 °C, using 0.5× TBE buffer (0.09 M Tris-borate, 0.09 M boric acid, 0.002 M EDTA).

2.3. Troubleshooting

Two main problems can occur:

(a) Agarose plugs become brownish following incubation with lysis buffer: there is

polyphenol contamination in the isolated nuclei.
Solutions: increase the concentration of PVP–40 and dietylether in the HB 1× nuclei extraction buffer, add 0.1% ascorbic acid to the HB 1× nuclei isolation buffer, repeat homogenate filtration and/or centrifugation at low *g* speed (step 2).

(b) BAC DNA inserts are smaller than 100 kb: it is due to incorrect high-molecular-weight DNA size fractionation.

Solution: Change the conditions of pulsed-field gel electrophoresis migration.

3. Typical results obtained

If the described protocols are followed:
– pulsed-field gel electrophoresis of partially-digested HMW *Musa* DNA occurs (*figure 1*),
– the NotI digestion of *Musa* BAC clones (*figure 2*) reveals inserts on average > 100 kb in size.

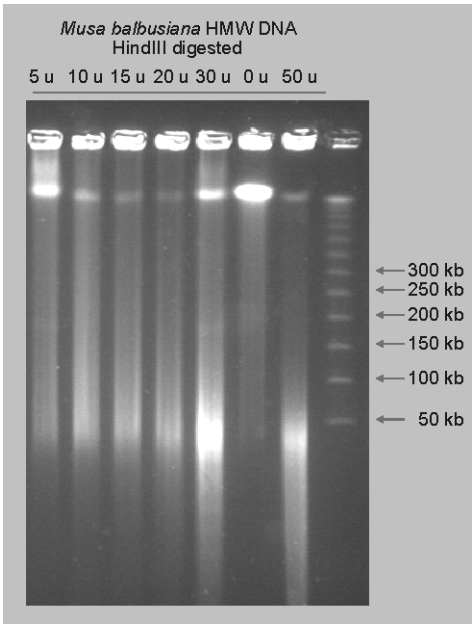

Figure 1.
PFGE analysis of partially digested *Musa balbisiana* HMW DNA. High-molecular-weight (HMW) DNA was digested with increasing concentrations of the restriction enzyme *Hind* III and separated using pulsed-field gel electrophoresis (PFGE) on 1% agarose gel in 0.5 × TBE, 6 V·cm^{-1}, switch time from (1 to 50) sec, angle 120°, for 20 h at 14 °C. The molecular weight standard is the lambda ladder PFGE marker (New England Biolabs). DNA digested with 50 units of *Hind* III represents a complete digestion of HMW DNA.

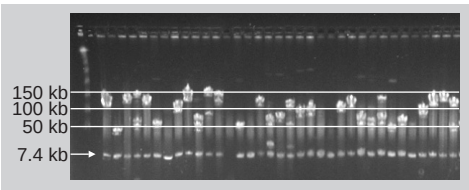

Figure 2.
Insert size analysis of randomly chosen clones from *Musa acuminata* Cavendish banana BAC library. BAC DNA was digested to release insert with NotI enzyme and separated by pulsed-field gel electrophoresis (PFGE) on 1% agarose gel in 0.5 × TBE, 9 V·cm^{-1}, switch time from (5 to 15) sec, angle 120°, for 5 h at 14 °C. The DNA was stained with ethidium bromide. The molecular weight standard is the lambda ladder PFGE marker (New England Biolabs). The 7.4-kb band represents the pIndigoBAC-5 *Hind* III-Cloning Ready vector (Epicentre).

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