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Yeast and Bacterial Artificial Chromosome (YAC and BAC) Clones of the Model Legume *Lotus japonicus*

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Abstract

Lotus japonicus is a model legume with a small diploid genome (n=6), short life cycle and high transformation ability. As a step towards positional cloning of developmental genes in *L. japonicus* we cloned pieces of high molecular weight (HMW) DNA into yeast and bacterial artificial chromosomes vectors (YACs and BACs) and transformed them into their respective hosts. Standardized cloning and transformation conditions as well as verification of inserts by pulsed field gradient electrophoresis (PFGE) and Southern hybridization are described.

Keywords: Nodulation, physical mapping, plant genome, positional cloning, size selection, symbiosis

1. Introduction

Genome analysis and map based cloning require the isolation of large pieces of the target DNA (Bent et al., 1994; Martin et al., 1993; Song et al., 1995). A major impediment for genome analysis using plasmid or phage cloning systems

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was the low average insert size. An alternative came with the development of yeast artificial chromosomes (YACs; Burke et al., 1987). This technology consists of cloning large pieces of DNA into modified vectors that have all the essential sequences of native yeast chromosomes (Fig. 1). Once transformed into suitable yeast cells, these clones are maintained and carried over as native chromosomes. The biggest advantage is the almost limitless cloning capacity (Albertsen et al., 1992; Guzman and Ecker 1988; Larin et al., 1991; Heiter et al., 1990). This potentially facilitates easier chromosome walking and physical mapping. However, YACs show some disadvantages, mainly chimerism and instability, which mislead genome analysis (Libert et al., 1993; Green et al., 1991; Infante et al., 1995).

A solution to the problems associated with YACs was found with the development of bacterial artificial chromosomes (BACs; Shizuya et al., 1992) This system consists of cloning large fragments of DNA into an F-factor based plasmid vector and maintaining the clones as low copy artificial chromosomes in suitable bacterial host cells. Even though the cloning capacity of BACs is much lower than that of YACs (upper size limits of 300–350 kb as opposed to 1.5–2 megabases of YACs), BACs are supposed to overcome most of the inherent problems of YACs, by being more stable and "user-friendly" than YACs. BAC libraries have already been constructed in some important crop plants (Wang et al., 1995; Woo et al., 1995). These libraries have been used for *in situ* hybridization mapping of genomes (Jiang et al., 1995).

To help develop the molecular genetics of *Lotus japonicus* we resorted to standardizing conditions for the construction of both YAC and BAC clones. Contour clamped homogeneous electric field-pulsed field gradient gel electrophoresis (CHEF-PFGE; Schwartz and Cantor, 1984; Chu et al., 1986) was used to select digested high molecular weight (HMW) DNA of *L. japonicus* ecotype "Gifu" B129. This DNA was cloned into pYAC4 and pBeloBAC11 vectors which were then transformed into *Saccharomyces cerevisiae* strain AB1380 and *E coli* DH10B, respectively, to generate YACs and BACs. Advantage was taken of the knowledge of soybean DNA separated by CHEF-PFGE (Funke et al., 1993) and cloned into pYAC4 (Funke et al., 1994).

As a model plant *L. japonicus* ecotype "Gifu" B129 has many advantages (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1993). It has a haploid genomic size of approximately 400 megabase pairs (x=n=6), which corresponds to about 0.5 pg of DNA per cell. Isolation of DNA is easy. It is a perennial and has many large flowers which make emasculation easy. It has got a very high seed number of about 6,000 per plant. It is self-fertile and plants can be generated from cell culture. Transformation with *Agrobacterium tumefaciens* and *A. rhizogenes* is easy and efficient (Handberg et al., 1994; J. Stiller, unpublished data). Root nodules appear within one week after inoculation

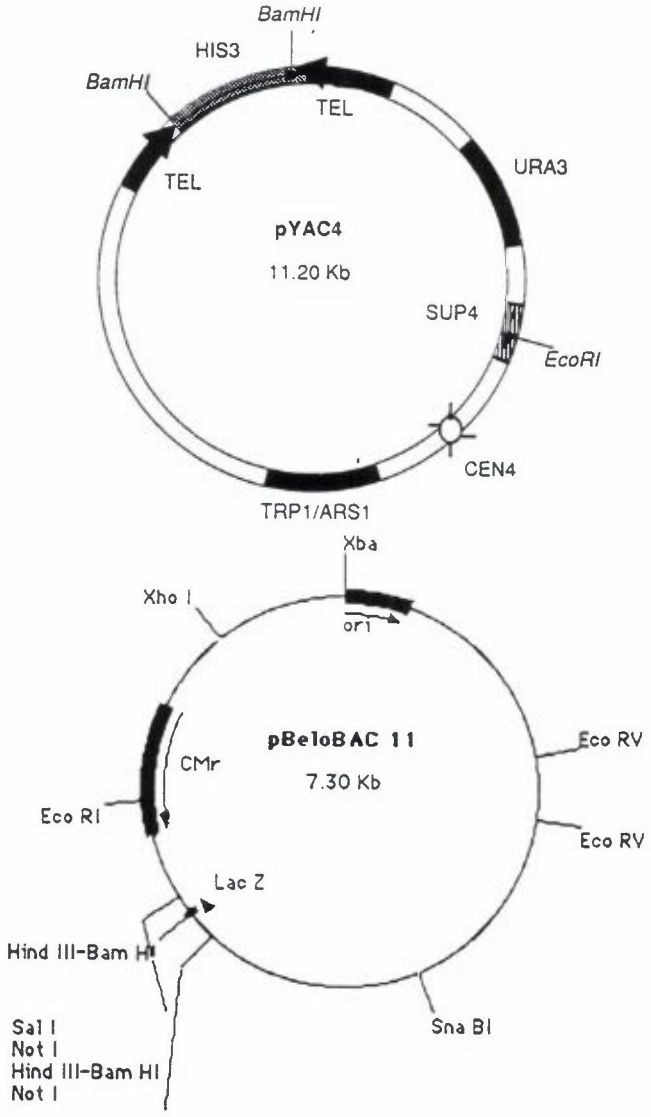


Figure 1. a) The pYAC4 vector into which YACs are cloned. The cloning site is an *Eco*RI site in the SUP4 gene in the plasmid. SUP4 is an amber suppresser gene that complements an adenine amber mutation in the host. Recombinant plasmids will bring back the amber color of the host. ARS2 – Autonomous replicating sequence that confers chromosomal level replication to the plasmid; CEN – Centromere sequence; TEL – Telomere seeding sequences; HIS3 – Spacer fragment, which when cleaved off by *Bam*HI gives rise to functional telomeres.
b) The pBeloBAC11 vector into which BACs are cloned. The cloning site is an *Hin*dIII site in the lacZ gene of the plasmid. The flanking *Not*I sites can be used to excise the insert.

with *Rhizobium loti* strains NZP 2213 and NZP 2037. Nitrogen fixation rates are good. Plants can be grown in closed tubes or plastic growth pouches. All these attributes make *L. japonicus* a legume of choice for the molecular genetic analysis of determinate nodulation. Another diploid *Medicago truncatula* has been suggested as a model legume for indeterminate nodulation (Barker et al., 1990).

2. Materials and Methods

Preparation of HMW DNA

Lotus japonicus ecotype Gifu B129 plants were grown in horticultural potting mix in the greenhouse for about 3 weeks. Twenty to thirty young leaves were picked and surface sterilized in 70% ethanol for 2 minutes. Then they were transferred into a small volume of protoplast buffer (36.4 g mannitol, 735 mg calcium chloride, 487 mg MES, 440 mg ascorbic acid, pH 5.6, in 500 ml water).

A digestion mixture of the following composition was prepared: 200 mg Driselase (Sigma O-0915); 100 mg Cellulase Onozuka R-10 (Serva 16419); 20 mg Pectolyase (Sigma P-3026) per 10 ml protoplast buffer, stirred for 30 minutes and adjusted to pH 5.6. This enzyme mix was briefly centrifuged and filter sterilized.

The leaves washed in protoplast buffer were cut with sterile razor blades into 1–2 mm strips in ice-cold protoplast buffer. The strips were incubated in 2–3 changes of cold protoplast buffer in 5 cm diameter Petri dishes. The buffer was then replaced with the already prepared digestion mixture, the Petri dish sealed with Parafilm® and placed on a rotating shaker at 60 rpm/room temperature for about 3 hours. The appearance of protoplasts was observed with a phase contrast microscope. When they looked round with very little cell debris and breakage, the incubation was stopped.

The suspension was then filtered through a 43 μm Nylon filter into a 50 ml plastic centrifuge tube. The protoplasts were counted using a haemocytometer and the concentration was brought to 10^8 per ml by centrifugation and resuspending in the desired volume. An equal volume of 1% low melting agarose (Gibco-BRL, USA) in protoplast buffer was added after the agarose had cooled down to 50°C. The mixture was then poured into prechilled plug molds. Once solidified, the plugs were extruded into a 5 cm Petri dish and incubated overnight at 50°C in lysis buffer (0.5 M EDTA, pH 9.3; 1% Sarkosyl: 0.1 mg/ml proteinase K) after which it was replaced with fresh lysis buffer and reincubated for a further 24 hours. Then the buffer was replaced with the

storage buffer (0.5 M EDTA, pH 9.3, 1% Sarkosyl) and stored at 4°C. Each plug thus prepared contained about 4 µg DNA.

Estimation of enzyme levels for optimum partial digestion

Four protoplast plugs were dialysed once against TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) for 15 min, twice against TE plus 1 mM PMSF (phenylmethyl sulphonyl fluoride) at 50°C for 5 min each and then once more against TE for 15 min. Two of the plugs were then equilibrated with 1x *Eco*RI buffer (NEB, USA) and the other two with 1x *Hin*dIII buffer (NEB, USA) each for 30 min on ice in Eppendorf tubes. Each plug was then cut into four smaller plugs. Each small piece was incubated with different amounts of enzyme – 0.05, 0.5, 5, and 15 units of *Eco*RI (NEB, USA) / 2, 10, 20, and 50 units of *Hin*dIII (NEB, USA) and 50 µl of the respective 1x buffer. The tubes were allowed to sit on ice for 30 more minutes for enzyme infusion and then incubated at 37°C for 1 hour. After 1 hour the reaction was stopped by adding 100 mM EDTA with 1 mg/ml of proteinase K. The tubes were left at 37°C for an additional 30 minutes. The plugs were loaded into a 1% low melting agarose gel along with a 50–1,000 kb Pulse marker (Sigma, USA). The gel was subjected to pulsed field gel electrophoresis in a CHEF DR II system (BioRad, USA) with a switch time of 10–20 seconds at 150 V/4°C for 16 hours. The gel was then stained with ethidium bromide (1 µg/ml) and viewed under UV. An estimate of the optimum enzyme concentration was made based on the varying degrees of concentration of HMW DNA in each of the compression zones (>200 kb) as opposed to a control undigested sample (Fig. 2).

Partial digestion and size selection by PFGE

For YAC clones – As above, two protoplast plugs were washed and then equilibrated with 1x *Eco*RI buffer. Each plug was cut into four equal portions which were then incubated with 0.02, 0.05, 0.08, and 0.1 units of *Eco*RI. Digestion was carried out as above, and the plugs were loaded into a 1% LMP agarose gel with a 25 mm long middle well. The gel was then subjected to PFGE under the same conditions as above. The sides of the gel were cut off, stained with ethidium bromide and viewed under UV to locate the compression zone of HMW DNA. The gel pieces were then realigned with the rest of the gel and a thin slice of gel, not more than 5 mm in width, corresponding to the compression zone, was cut off. This slice contained the large DNA fragments needed for ligation.

For BAC clones – The same methodology as for YACs was followed for the BACs, but with differences in enzyme concentrations and pulse time. The plugs

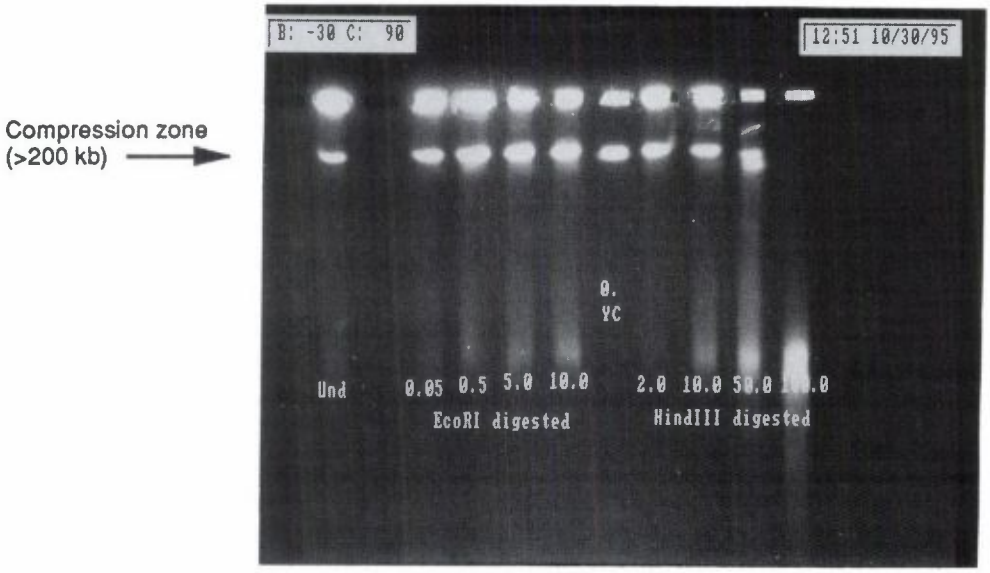


Figure 2. Optimization of enzyme levels for partial digestion by PFGE. The protoplast plugs were digested with different quantities of *Eco*RI and *Hin*dIII and subjected to PFGE. The intensity and size of the compression zone just below the wells where the HMW DNA concentrates was taken as an indication of partial digestion. From the figure it can be seen that 0.5 units of *Eco*RI and 2 units of *Hin*dIII gives the best partial digestion. However, for the construction of clones, a range of enzyme levels close to these optima were used for partial digestion. Und – undigested; YC – yeast chromosomes.

were incubated with 1, 2, 3, and 4 units of *Hin*dIII in 1x NEB 2 buffer. The gel was subjected to PFGE with a switch time of 60–90 seconds and a gel slice corresponding to the 200–300 kb range was cut off. This slice was equilibrated in TE for about 2 hours at 4°C. The slice was then put into a 1.5 ml Eppendorf tube and melted at 65°C for 20 minutes, then it was transferred to 40°C. After 10 minutes beta-agarase enzyme (Epicentre, USA) was added at 2 µl per 200 µl agarose and the tube was incubated for 3 hours. This DNA solution was used directly for ligation.

Isolation and preparation of the vectors

The YAC vector: pYAC4 (Fig. 1a) – The vector was prepared from *E. coli* HB101 by a normal maxi-prep procedure using the alkaline lysis method (Sambrook et al., 1989). The isolated vector was purified twice by continuous

gradient equilibrium centrifugation in cesium chloride-ethidium bromide gradients (Sambrook et al., 1989) using a Beckman Ti50 rotor (Beckman, Illinois, USA) at 45,000 rpm for 48 hours. About 50 µg vector DNA was subjected to double digestion, first with 30 units of *Bam*HI and then with 30 units of *Eco*RI. The digested vector was dephosphorylated using shrimp alkaline phosphatase (USB, Ohio, USA) at a rate of 1 unit/µg of plasmid DNA. This double digested dephosphorylated DNA was used for ligation.

The BAC vector: pBeloBAC11 (Fig. 1b) – A single colony of *E. coli* DH10B with pBeloBAC11 vector was inoculated into 10 ml LB medium containing 12.5 µg/ml chloramphenicol and was grown overnight at 25°C shaking at 250 rpm. This culture was used to inoculate 3 liters LB supplemented with 12.5 µg/ml chloramphenicol. This culture was grown for 24 hours at 25°C shaking at 250 rpm. The plasmid was isolated using Qiagen's Maxi plasmid isolation kit (Qiagen, USA). The isolated vector was purified by plasmid-safe DNAse (Epicentre, Wisconsin, USA) treatment and by cesium chloride-ethidium bromide equilibrium centrifugation at 48,000 rpm for 48 hours using a fixed angle rotor 70.1 (Beckman, Illinois, USA). The plasmid was then digested to completion with *Hin*dIII and dephosphorylated using shrimp alkaline phosphatase at 30°C for 1 hour at the rate of 1 unit/µg of plasmid. The phosphatase was heat-inactivated at 70°C for 20 minutes.

Ligation

YAC ligation – The gel slice with large DNA fragments was equilibrated with the equilibration buffer (66 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 30 mM NaCl, 0.75 mM spermidine, 0.30 mM spermine) at room temperature (about 22°C) for 30 min. The equilibrated gel slice was cut into 2–3 mm sections. About 200 mg of these slices were weighed out into an Eppendorf tube. Vector DNA (2 µg) was added to this tube (molar volume of 10:1 in vector excess). The agarose slices were melted at 68°C for 20 min. The tube was then cooled to 37°C for 10 min and 1/10 volume of 10x T4 ligase buffer (prewarmed to 37°C) was added. After mixing the contents gently by rotating the tube and incubating at 37°C for a further 10 min, 1600 units of T4 DNA ligase were added. Gentle mixing was repeated and the tube left at 37°C for 45 minutes for the enzyme to diffuse. The ligation was left to proceed at room temperature overnight. The ligation mixture was melted at 68°C for 20 minutes. About 100 µl of the melted mixture was put in a new tube (with a cut pipette tip), transferred to 40°C, and after 10 minutes 2 µl of beta-agarase enzyme was added. The digestion of agarose was allowed to proceed for 3–4 hours. The completely aqueous solution was then dialysed overnight against 1x TE in an Ultrafree-MC filter tube (Millipore, Massachusetts, USA) at 4°C.

BAC ligation – The ligation was carried out in a 100 µl volume in a molar ratio of 10:1 in vector excess (144 ng of size-selected *L. japonicus* ecotype "Gifu" DNA was ligated to 36 ng of digested dephosphorylated vector – in terms of volume 48 µl of *Lotus* DNA was ligated to 1 µl of vector). Six hundred units of T4 DNA ligase (NEB, Massachusetts, USA) were used and the reaction was carried out overnight at 16°C. Before transformation the ligation was dialyzed against 1x TE in an Ultrafree-MC filter tube (Millipore, Massachusetts, USA) at 4°C overnight.

Transformation

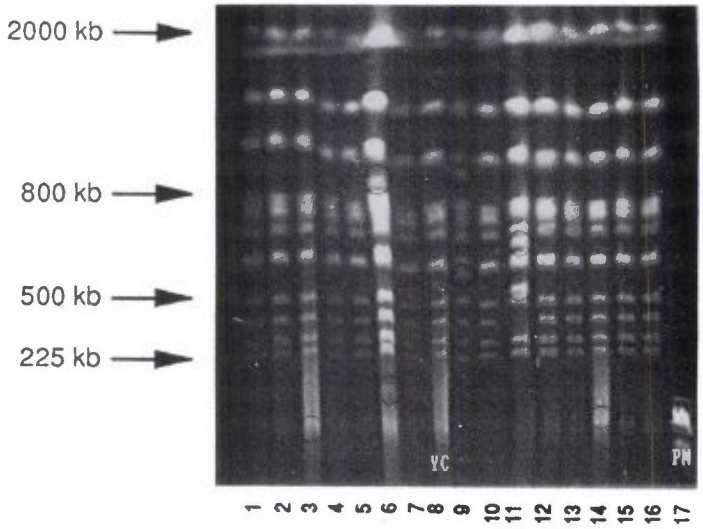
Transformation of host yeast strain AB1380 was carried out by spheroplasting. About 200 ml of culture grown in YPD media was harvested when the cell density reached 3×10^7 cells/ml. The culture was divided into 4 tubes of 50 ml each. They were then spun down and washed first with distilled water and then with 1 M sorbitol. The cells were then resuspended in 20 ml of spheroplasting buffer (1 M sorbitol, 10 mM sodium phosphate, 10 mM EDTA, pH 8, 30 mM ß-mercaptoethanol). Thirty, 50, 75, and 100 microliters of 500 mg/ml lyticase (ICN, USA) were added to the different tubes. Spheroplasting was carried out at 30°C for 10 min. The extent of spheroplast formation was judged by spectrophotometric reading at 800 nm. The batch of cells showing 60–70% spheroplasting was selected for transformation. Cells were spun down at 900 rpm for 5 minutes in a Labofuge-B (American Scientific Products, USA). The pellet was washed in 20 ml SSP (1 M sorbitol, 10 mM sodium phosphate). The pellet was then resuspended in 20 ml YPD buffer (Ausubel et al., 1995) in 1 M sorbitol. The tube was then gently shaken on sides at 30°C for 30 min. Ten ml of STC (1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) was added to this solution and the cells were then harvested at 900 rpm for 5 min. The cells were then resuspended in 2 ml STC. These cells were used for transformation. Ligation mix (50 µl) was added to 300 µl of the competent cells for each set of transformation (one 50 ml culture tube). The tubes were left at room temperature for 10 min. Then 3 ml PEG solution (20% PEG 8000-Sigma, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) was added to this tube with just enough force to resuspend the cells. The tubes were left at room temperature for a further 10 minutes. Spheroplasts were then pelleted at 900 rpm for 4 min. The pellet was resuspended in 450 µl of SOS (1 M sorbitol, 6.5 mM $CaCl_2$, 0.25% yeast extract, 0.5% bactopeptone, uracil 20 mg/ml, tryptophan 20 mg/ml, pH 5.8) and incubated at 30° C for 20 min. Then 10 ml top agar (0.8% Difco in YPD; 45–48°C) was added to each tube. Cells were mixed with the top agar by inverting the tube 2–3 times. The mixture was then plated on single selection plates (182 g sorbitol, 20 g agar, 20 g dextrose, 0.8 g -URA dropout powder BIO 101 Inc., 1.7 g

yeast nitrogen base w/o amino acids and ammonium sulphate, 5 g ammonium sulphate in 1,000 ml total volume) and left at 30°C for 2–3 days.

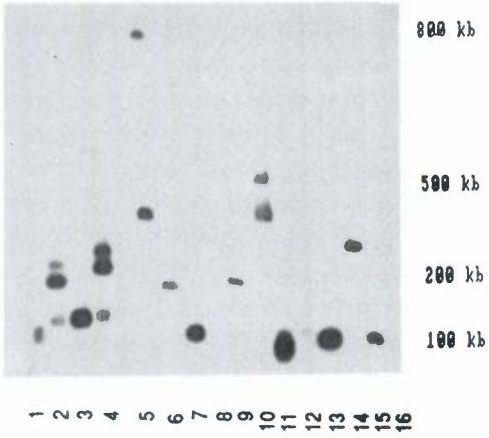
BAC transformation of competent *E. coli* DH10B (Gibco-BRL, USA) was carried out by electroporation using an *E. coli* PULSER (Bio-Rad, USA) at the following settings: voltage 2.5 kV, capacitance 25 μF, resistance 100 ohms. Ligation mix (2 μl) was added to 20 μl of competent cells and then the pulse was applied. After electroporation the cells were transferred to 1 ml SOC solution (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0) and incubated at 37°C, 200 rpm for 45 min. The cells were then spread on LB plates containing chloramphenicol (12.5 μg/ml), X-gal (40 μg/ml), and IPTG (0.072 μg/ml). The plates were incubated at 37°C for 24 hrs.

Insert size check

YACs: The yeast colonies that appeared on the single selection plates were picked on to double selection plates (same as single selection plates except in that it contained -URA-TRP dropout powder instead of -URA powder). After incubation at 30°C for another 3 days about 20 of those colonies that turned red, were picked up for an insert size check. Each of these colonies was inoculated into 5 ml of 1.5x AHC (2x AHC- 3.4 g YNB, 10 g ammonium sulphate, 20 g casein-hydrolysate acid, 40 mg adenine hemisulphate, 100 μl concentrated HCl, 40 g dextrose) containing 50 mg/ml ampicillin. The cultures were shaken at 30°C/250 rpm till the cultures turned fully red (about 48 hours). Cells were spun and then washed with 5 ml SE (0.9 M sorbitol, 50 mM EDTA). The washed cells were resuspended in 150 μl LMP agarose containing 0.25 mg/ml lyticase and 30 mM ß-mercaptoethanol. Each of the cultures was then put into pre-chilled plug molds. Once solidified the plugs were extruded into separate Eppendorf tubes containing 1.5 ml spheroplasting solution (0.9M sorbitol, 50 mM EDTA, 30 mM ß-mercaptoethanol and 0.25 mg/ml lyticase). The tubes were incubated at 37°C overnight. The spheroplasting solution was then replaced with ESP (0.5 M EDTA, pH 8.0, 1% Sarkosyl, 1 mg/ml proteinase K). Incubation was done overnight at 50°C. The old solution was replaced with fresh ESP and incubation was continued overnight at 50°C. Small pieces of the treated plugs thus obtained were subjected to PFGE in a 1% agarose gel at 200 V/4°C with a 20–70 seconds switch time for 16 hours. The gel was stained with ethidium bromide (1 μg/ml) and viewed under UV to check the insert sizes of the artificial chromosomes (Fig. 3a). For confirmation the gel was blotted on to a Zeta-Probe® GT Nylon membrane (Bio Rad, CA, USA) and the membrane probed with ^{32}P labelled pBR322 (which has high sequence homology to the YAC vector) as well as *Lotus japonicus* ecotype Gifu genomic DNA.



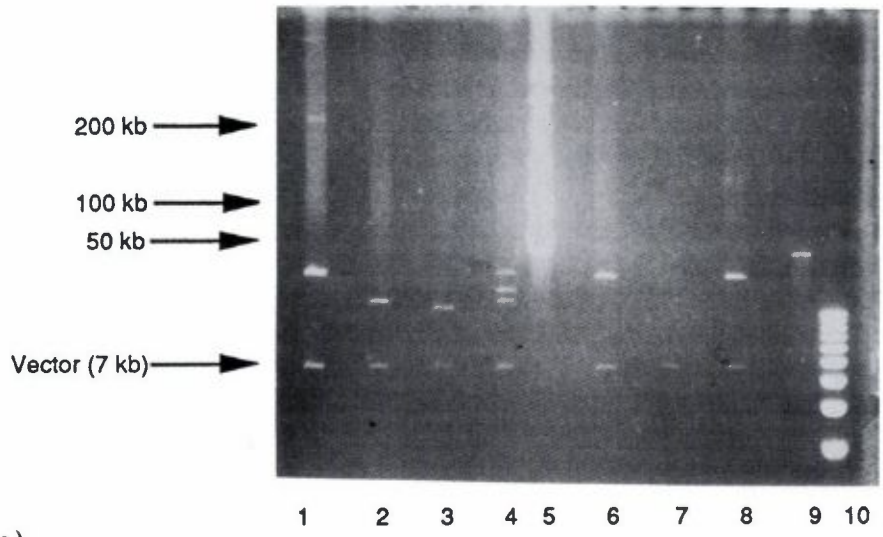
a)



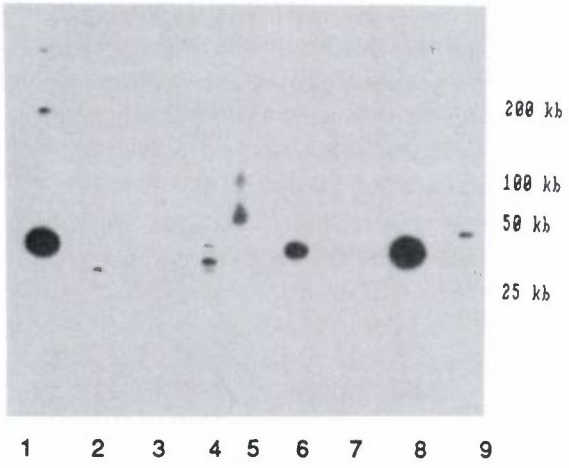
b)

Figure 3. a) PFGE of recombinant yeast DNA to separate the individual chromosomes and check for the visible YACs. Lanes 1–7 and 9–16 has the recombinants. Lane 8 – YC has the 16 native yeast chromosomes, the smallest being 225 kb and the largest 2200 kb. Lane 17 – PM has a 0.1–200 kb size standard. Putative YACs are circled in black. The presence of more than one YAC in some lanes may be due to mixing up of colonies or multiple YAC transformations. In Lane 11 a native yeast chromosome (365 kb) is missing and is probably appearing as a larger chromosome (500 kb or 600 kb) further up suggesting possible recombination with a YAC. The sizes of the YACs vary from >800 kb (lane 6) to about 100 kb (lane 15).

b) Southern blot of YAC gel probed with radiolabelled pBR322 DNA and autoradiographed. This plasmid has very high sequence homology to the YAC vector pYAC4 and hence hybridizes specifically to the YACs. The relative sizes are given on the right. Some lanes show multiple YACs. Lane 8 has the native yeast chromosomes while lane 17 has the 0.1–200 kb marker.



a)



b)

Figure 4. a) PFGE of recombinant BAC plasmids isolated from white colonies and digested with *Not*I. Lanes 1–4, 6, 8 and 9 have the recombinant clones. Lane 5 has a 50–200 kb size marker. Lane 7 has the 7 kb BAC plasmid while lane 10 has the 1 kb ladder. The sizes of the inserts vary from >200 kb (lane 1) to about 25 kb (lane 3).

b) Southern blot of BAC gel probed with radiolabelled *L. japonicus* DNA and autoradiographed. The relative sizes are given on the right. Lanes 1–4, 6, 8 and 9 have the recombinant clones. Lane 5 has a 50–200 kb size marker. Lane 7 has the 7 kb BAC plasmid. The lack of a signal in lane 3 probably indicates that it is a low copy sequence.

Hybridizations were carried out in The Belly Dancer® hybridization water bath (Stovall Life Sciences Inc., NC, USA). The hybridization solution contained 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2 and 7% SDS. The hybridizations were carried out at 60°C for 20 hours. The membranes were washed twice in Wash I (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2 and 5% SDS) and twice in Wash II (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2 and 1% SDS). Each wash was done at 60°C for 20 min. The membranes were autoradiographed (Fig. 3b shows autoradiogram of the vector probe). For use as probe *L. japonicus* ecotype "Gifu" DNA was isolated according to Dellaporta et al. (1983).

BACs: White colonies containing putative inserts were picked on to a new LB plate for a second color screen. The clones were then transferred to 96 well microtiter plates containing 60 µl of freezing buffer (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% v/v glycerol, 12.5 µg/ml chloramphenicol in LB). The plates were then stored at –80°C. About 10 clones from each transformation were checked for insert size. The check was done by a normal miniprep followed by a *Not*I digestion. *Not*I has restriction sites very close to the cloning site on either side. Being a rare cutting enzyme few cutting sites were expected within the insert DNA. About 5 µl plasmid solution was digested in a total reaction volume of 20 µl for about 4–5 hours. The restricted plasmid DNA was then subjected to PFGE at 150 V with a 10 second switch time for 16 hours at 4°C in a 1% agarose gel with appropriate size markers. The gel was stained and viewed as before (Fig. 4a). The gel was then blotted on to a Nylon membrane and probed with ^{32}P labelled total *L. japonicus* "Gifu" genomic DNA for confirmation. Hybridization was carried out exactly as before (Fig. 4b).

3. Results

It was possible to produce and characterize YAC and BAC clones for the model legume *Lotus japonicus*. We were successful in isolating relatively undegraded DNA from *L. japonicus* protoplasts. This step was critical in the construction of the clones carrying high molecular weight DNA. Secondly, we standardized the amount of enzyme needed for optimum partial digestion. From our trials we found that 0.02 units of *Eco*RI and 2 units *Hin*dIII were optimal for digestion (Fig. 2). However, since it was difficult to estimate the exact quantity of enzyme needed, we digested the DNA with a range of enzyme units close to the quantity estimated to be optimal. Thus, we digested the DNA with 0.02, 0.05, 0.08, and 0.1 units of *Eco*RI and ran them all together for size selection. Likewise, we digested the DNA with 1–4 units of *Hin*dIII. We then isolated the desired size digested DNA fragments which we later used for

ligation. However, we found that some lower sized DNA fragments remained entrapped in the compression zones. This was found when we checked the insert sizes of the clones. Some of them appeared smaller than expected (Fig. 3a). This compares well with findings from soybean DNA separated by PFGE (Funke et al., 1994, unpublished data). We also standardized the amount of vector needed for optimum ligation in terms of the volume of the compression zone so that we did not have to measure the amount of DNA in the compression zone every time. We optimised conditions for the transformation of yeast and bacteria with high molecular weight *L. japonicus* DNA. Interestingly, while checking our YAC clones by hybridizing with *L. japonicus* DNA, we found that it hybridized with some of the larger yeast chromosomes, suggesting unexpected homology between yeast and *L. japonicus*. In one of the transformants we found that one of the native chromosomes had shifted to a new position of larger size (Fig. 3a, lane 11) suggesting possible recombination with a YAC.

The transformation efficiency of yeast AB1380 was found to be 1.6×10^4. However, the efficiency was found to decrease about 10 fold when transformed with YACs. The yeast colonies that appeared in the single selection plate were divided into small, medium and large based on their sizes. Of these only 13.5% of the small colonies turned red on double selection plates, while >60% of the medium and large colonies turned red. Altogether we obtained about 250 clones with an average size of 125 kb.

The transformation efficiency of *E. coli* DH10B was found to be about 0.7×10^7. However, as in case of the yeast transformation efficiency was found to drop >10 fold when transformed with BACs. More than 95% of the white colonies stayed white after a second screening. We got about 400 clones (only 200 µl of the total 1 ml transformation volume was plated) with an average size of 60 kb.

4. Discussion

YAC and BAC clones were derived from genomic DNA of *Lotus japonicus*. The procedures presented here gave rather low insert sizes, which may require optimization, for example, by using microbeads instead of agarose plugs. However, existing YACs and BACs provide the initial tools for further genome analysis such as the mapping of YACs/BACs.

High molecular weight DNA cloning requires very careful manipulation of native DNA. One of the critical initial steps is the isolation of intact DNA. Estimation of enzyme concentration for optimum partial digestion is another critical factor. Assuming that the number of protoplasts or nuclei embedded are

the same, this concentration can, to a certain extent, be calculated from the size of the genome. The size selection, ligation and transformation steps would be nearly the same for all genomes. It should be noted that more size selections can be done before transformation with a proportional loss of DNA. Moreover, the transformation efficiency decreases with increasing insert sizes, as shown above. We also observed that many yeast clones harbored more than one YAC. Often the additional YACs were not visible on the ethidium bromide stained PFGE gels. These multiple hybridization signals may represent YAC instabilities, multiple transformations or insertion of YACs or YAC fragments into native yeast chromosomes. About 50% of the tested YACs showed more than one signal after hybridization to the vector.

With the prospect of *L. japonicus* being accepted as a model plant for determinate nodulation, the need for properly represented HMW DNA libraries can arise anytime in the near future. The first steps towards this are reported here. Insert sizes and clone numbers need to be increased. Accepted model organisms, like *Arabidopsis thaliana* (Ward and Jen, 1990; Grill and Somerville, 1991; Schmidt et al., 1992) and many crop species (Klein et al., 1994; Del Favero et al., 1994; Edwards et al., 1992; Martin et al., 1992; Ronald et al., 1995; Wing et al., 1995) already have well characterised HMW DNA libraries and many genes have been isolated from the same (Ronald et al., 1995; Klein et al., 1994; Zhang et al., 1994; Martin et al., 1992; Ward and Jen, 1990). Recently, it has been shown that YAC clones can be transferred into host plant cells and checked for functional complementation (Van Eck et al., 1995).

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Abbreviations

BAC – Bacterial Artificial Chromosome; CHEF-PFGE – Contour Clamped Homogeneous Electric Field-Pulsed Field Gradient Electrophoresis; HMW – High Molecular Weight; YAC – Yeast Artificial Chromosome.

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