

Research Note

A Bacterial Artificial Chromosome Library of *Lotus japonicus* Constructed in an *Agrobacterium tumefaciens*-Transformable Vector

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We constructed a BAC library of the model legume *Lotus japonicus* with a 6-to 7-fold genome coverage. We used vector PCLD04541, which allows direct plant transformation by BACs. The average insert size is 94 kb. Clones were stable in *Escherichia coli* and *Agrobacterium tumefaciens*.

Additional keywords: Arabidopsis, functional genomics, map-based cloning, symbiosis.

Lotus japonicus is a model legume that is suitable for functional genomic analysis of *Rhizobium* spp.-induced nodulation and mycorrhiza associations (Handberg and Stougaard 1992; Jiang and Gresshoff 1997; Wegel et al. 1998). The plant is characterized by a relatively small genome (approximately 450 Mb), six chromosomes, and a high transformation efficiency (Stiller et al. 1997). It is the subject for extensive analysis involving T-DNA and transposon-mediated insertion mutagenesis (Schauser et al. 1999), promoter-trapping (Martirani et al. 1999), and the collection of chemically induced mutants (Szczyglowski et al. 1998; Wegel et al. 1998). The development of a molecular map (Jiang and Gresshoff 1997) and the characterization of expressed sequence tags (ESTs) (Asamizu et al. 2000; Szczyglowski et al. 1997) have opened the possibility of gene discovery and functional genomics in this model legume.

The construction of plant BAC libraries is now becoming a relatively routine procedure because of improvements to DNA isolation and cloning techniques. Furthermore, it has been reported that a new generation of vectors are able to deliver a BAC insert directly into the plant genome via *Agrobacterium* spp.-mediated transformation (Hamilton et al. 1999; Liu et al. 1999). This provides new possibilities to introduce intact complex genomic structures such as quantitative trait loci (QTLs) and multigene families into the plant (Michelmore 1996).

Here we describe the characteristics of a new *L. japonicus* BAC library. The library was constructed with partially *Hin*-III digested DNA of ecotype "Gifu." The plant nuclei were used as a DNA source and PCLD04541 as a cloning vector. PCLD04541 is a binary vector (Bent et al. 1994) capable of the stable maintenance of very large plant DNA fragments in *Escherichia coli* (Tao and Zhang 1998; Merksen et al. 2000). Before cloning, two rounds of DNA size selection were performed. Resulting HMW DNA sample was ligated with digested and/or dephosphorylated vector and introduced into *E. coli* by electroporation. The library was arrayed in 87 384-well microtiter plates (33,408 clones total) and spotted in a duplicated manner onto nylon membranes for hybridization-based analysis. We digested 111 BAC clones with *Not*I (Fig. 1). The average size of the BACs was 94 kb. About 40% of the clones were approximately 80 kb, 27% were between 120 and 260 kb, and about 10% were smaller than 50 kb. No "empty" clones (i.e., no plant DNA insert) were found. The probability of finding any sequence of interest within the library would be $P = 1 - (1 - L/G)^N$, where L is the average length of the clone insert, G is the haploid genome size of organism, and N is the number of clones in the library (Clarke and Carbon 1976). On the basis of this formula and an estimated 450 Mb genome size, the probability of finding any *L. japonicus* sequence in our library is 99.91%.

In order to estimate the number of clones containing chloroplast DNA, we hybridized the library with the photosystem II gene *psbA* from pea (the *psbA* DNA sequence is highly conserved among numerous plant species) (Fig. 2). A total of 193 duplicated BAC clones that were positive for the *psbA* were obtained. This provides a very low content (0.58%) of chloroplast DNA for the library.

DNA pools, each representing one 384-well plate, were prepared for polymerase chain reaction (PCR) screening. Because the library will be used primarily in the area of symbiosis genomics, we characterized it by screening with several symbiosis-specific probes from *L. japonicus*. PCR tests were developed for four ESTs and genes related to nodulation: AF000394 (late nodulin EST library cDNA similar to yeast adenosylsuccinate synthetase) (Szczyglowski et al. 1997),

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LjNPP2C1 (protein phosphatase 2C gene induced during root nodule development) (Kapranov et al. 1999), a nodule-specific tag *CHEETAH* (J. Stiller, *personal communication*), and nodule inception gene *NIN* (Schauser et al. 1999) (Fig. 3A). To estimate the number of individual BAC clones corresponding to one PCR-positive pool and/or plate, we performed a Southern hybridization analysis of a plate positive for the *CHEETAH* PCR test. The analysis revealed a single positive clone (Fig. 3B). These results, along with the knowledge that the rest of the analyzed probes are single-copy genes, shows an acceptable correlation between the number of PCR-positive plates per probe and the estimated 6- to 7-fold genome coverage of the library.

We also tested the possibility of use of Arabidopsis probes for *L. japonicus* genomics. We chose a set of 14 putative Arabidopsis protein kinases (provided by J. Botella of the University of Queensland) that have no apparent DNA homology to one another. Probes were amplified from plasmid templates and hybridized to the BAC filters with lower stringency washing conditions (55°C rather than 60°C). Approximately

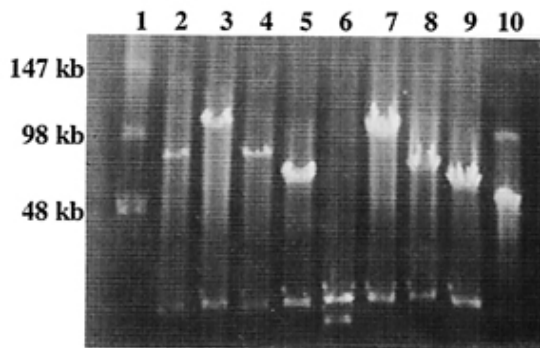


Fig. 1. Size characterization of eight large DNA inserts of *Lotus japonicus* cloned in the PCLD04541 vector. DNA was isolated with alkaline lysis and subjected to *NotI* digestion followed by pulse-field electrophoresis. Clone sizes are between 70 and 110 kb (lanes 2-5 and 7-9), except for lane 6, which is approximately 20 kb. Molecular markers (lanes 1 and 10) are lambda concatemers. One hundred and eleven BAC clones were characterized by this analysis.

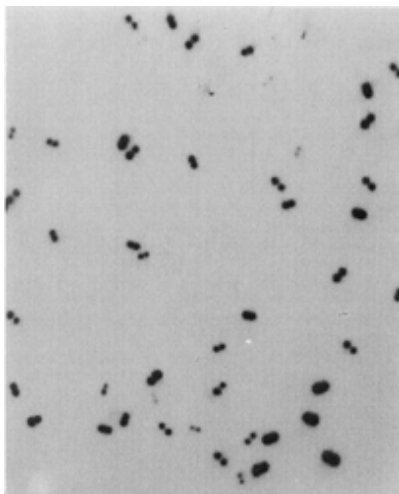


Fig. 2. Southern hybridization of the BAC filters with the *psbA* pea chloroplast probe. Result obtained for the nylon filter containing BACs from plates 37-73 is shown.

250 single and 75 duplicated signals were determined (data not shown). Out of the duplicated clones, 23 BACs produced very strong positive signals and 52 BACs gave moderate but reliably positive signals. In average, this provided 5.4 positive duplicated BACs per one Arabidopsis kinase.

BAC clones ranging from of 40 to 140 kb were transferred into electrocompetent *Agrobacterium tumefaciens* strains 4404, AGL1, and GV3101. AGL1 and GV3101 are capable of

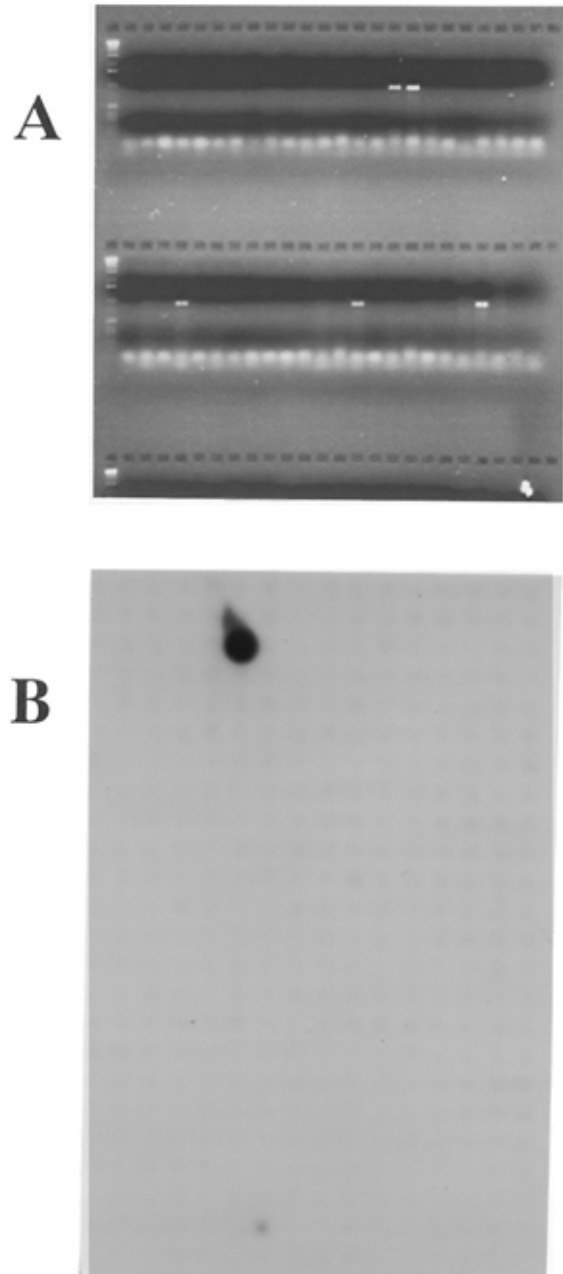


Fig. 3. Polymerase chain reaction (PCR) and hybridization screening of the library with probes for nodulation-specific genes. **A**, PCR result for the AF000394 expressed sequence tag probe. PCR products were separated in 1.5% agarose. Five positive signals (two for the upper lane, three for the lower lane) were found. PCR products were sequenced directly to confirm the test. **B**, Southern hybridization analysis of the 384-well plate that was PCR positive for the *CHEETAH* nodule-specific promoter.

maintaining intact BACs because fingerprints of clones re-introduced from AGL1 and GV3101 back into *E. coli* were identical to fingerprints of original clones propagated in *E. coli* (Fig. 4). The transformation of 4404 strain resulted in instability and the rearrangement and/or truncation of large BAC inserts (data not shown), probably because this strain is not recombination deficient.

BAC libraries have become one of the most powerful resources in the genomics of various organisms, including plants. Plant BAC libraries are being used for map-based cloning of single loci as well as the generation of long contigs and physical maps. The most advanced use of BACs in whole-genome analysis has been demonstrated for Arabidopsis and rice (Bevan et al. 1998; Hong et al. 1997; Sato et al. 1998), but these systems are not suitable for the study of symbiotic nitrogen fixation and mycorrhizal associations. For latter purposes, the legumes *L. japonicus* and *Medicago truncatula* have become model plant systems because of relatively small genome sizes, true diploidy, and high efficiency of transformation (Cook et al. 1997; Stiller et al. 1997; Wegel et al. 1998). Recently, a *M. truncatula* BAC library containing approximately five haploid-genome equivalents has been constructed by Nam et al. (1999). Here we report a new, 6- to 7-fold BAC library for *L. japonicus*. The advantage of our library is that it has been cloned in a binary vector that allows

direct transformation into *A. tumefaciens* and therefore allows the introduction of an intact BAC insert into the plant genome.

We suggest that the new *L. japonicus* BAC library will be useful for the entire legume research community. The lack of genes isolated by map-based cloning in legumes could be attributed mainly to the deficit of large-insert libraries. The use of the new transformation-ready BAC library of *L. japonicus* reported here, along with genetic analysis of symbiosis-deficient mutants and development of molecular maps (Jiang and Gresshoff 1997), provide powerful possibilities for map-based cloning and the construction of a whole-genome physical map based on BACs.

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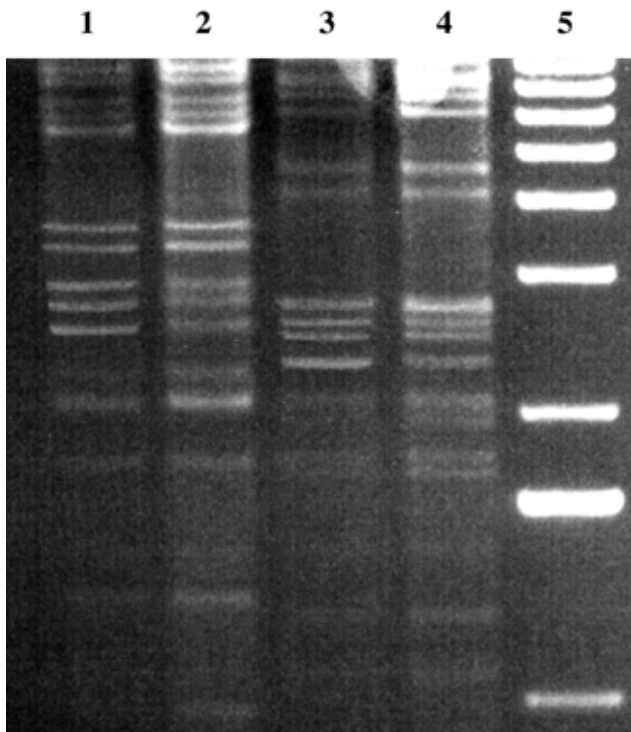


Fig. 4. *Lotus japonicus* BACs are stable in *Agrobacterium tumefaciens*. BAC clones were introduced into *A. tumefaciens* strains AGL1 and GV3101 and then isolated from *A. tumefaciens* and transferred back into *Escherichia coli*. BAC DNA was digested with *Eco*RI and fingerprinted by 1.2% agarose gel electrophoresis. BAC fingerprints in both cases were the same. Lane 1, BAC clone 1A12 (70 kb in size) isolated from *E. coli*; lane 2, 1A12 clone after transferring into AGL1 and back into *E. coli*; lane 3, BAC clone 1B14 (90 kb) isolated from *E. coli*; lane 4, 1B14 clone after transferring into GV3101 and back into *E. coli*; lane 5, 1 kb ladder (Life Technologies, Mulgrave, Australia). The smallest ladder band shown on this lane is 1,018 bp.

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