



## Construction and characterization of a common bean bacterial artificial chromosome library

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### Abstract

We have constructed a common bean (*Phaseolus vulgaris* L.) bacterial artificial chromosome (BAC) library consisting of 33 792 clones and an estimated 3- to 5-fold coverage of the common bean genome. Leaf nuclei were used as the source for high-molecular-weight DNA, and an endonuclease/methylase competition assay was employed to partially cleave the DNA. The library was screened with a number of nuclear and mitochondrial probes. Each nuclear probe identified at least two BACs with an average insert size of ca. 100 kb. Only 26 clones were identified after hybridizing with mitochondrial probes, indicating contamination with organellar sequences is low. Numerous clones could be identified after screening the library with two repetitive probes flanking the nuclear fertility restorer *Fr*. Intriguingly, 12 clones appeared to hybridize to both markers, and restriction analysis of these clones revealed that they can be assembled into maximally four contigs, suggesting that these repetitive probes may be useful for the physical mapping of the *Fr* locus.

### Introduction

Large insert libraries have made valuable contributions to genome analysis and molecular genetics. In plants, large insert libraries have been developed for several crop species, such as rice (Umehara *et al.*, 1995; Wang *et al.*, 1995), tomato (Martin *et al.*, 1992; Bonnema *et al.*, 1996), soybean (Marek and Shoemaker, 1997; Danesh *et al.*, 1998), maize (Edwards *et al.*, 1992), lettuce (Frijters *et al.*, 1997), and sorghum (Woo *et al.*, 1994). Clones obtained from such libraries have been used to facilitate gene isolation by positional cloning (Martin *et al.*, 1993; Buschges *et al.*, 1997), to study the relationship between physical and genetic distance (Civardi *et al.*, 1994), and to assess genome architecture (San Miguel *et al.*, 1996).

Today, the most commonly used system for constructing large insert libraries in plants is the bacterial artificial chromosome (BAC) system. The BAC vector is an F plasmid-based vector that is maintained as a single-copy plasmid in a recombination-deficient *Escherichia coli* host to promote sequence stability (Shizuya *et al.*, 1992). Although the insert size found

in most BAC libraries (up to 350 kb, averaging 100–150 kb; Shizuya *et al.*, 1992; Woo *et al.*, 1994; Wang *et al.*, 1995) is much smaller than the size found in some yeast artificial chromosome (YAC) libraries (over 600 kb; Umehara *et al.*, 1995; Bonnema *et al.*, 1996), the BAC system offers some important advantages over the YAC system. In contrast to YACs, BAC insert DNA is easy to isolate and manipulate and clone instability and chimeras, a particular problem in YAC libraries (Green *et al.*, 1991; Dunford *et al.*, 1993; Umehara *et al.*, 1995), has been reported to be low (Bent *et al.*, 1998) or absent (Shizuya *et al.*, 1992; Woo *et al.*, 1994; Wang *et al.*, 1995) in BAC libraries.

High-molecular-weight genomic clones from the common bean genome are of interest to us as we pursue two objectives: the construction of a complete physical map of the bean genome, and the positional cloning of two fertility restorer loci. Research in our laboratory focuses on nuclear-mitochondrial interactions in common bean, *Phaseolus vulgaris*, using a cytoplasmic male sterility (CMS) mutation. CMS is a non-lethal condition caused by mitochondrial DNA lesions, and restoration to fertility can be effected by the

action of nuclear fertility restorer genes (Schnable and Wise, 1998). Two independent fertility restorer genes, *Fr* and *Fr2*, have been identified in common bean (Mackenzie and Bassett, 1987; Mackenzie, 1991; He *et al.*, 1995a,b). Molecular marker-based genetic maps of common bean have been published (Vallejos *et al.*, 1992; Nodari *et al.*, 1993a). Common bean restriction fragment length polymorphism (RFLP) probes, together with random-amplified polymorphic DNA (RAPD) markers, have been used to position *Fr* and *Fr2* on the genetic map (He *et al.*, 1995b; Jia *et al.*, 1997). Only one fertility restorer gene has been cloned to date (Cui *et al.*, 1996), and little is known about the molecular mechanisms underlying fertility restoration in CMS systems. We have developed molecular markers closely linked to *Fr* (He *et al.*, 1995b, and unpublished results), and one of our objectives is to isolate *Fr* through map-based cloning.

Legume species become genetically better characterized as is evidenced by publications of several molecular marker-based maps (Keim *et al.*, 1990; Vallejos *et al.*, 1992; Menancio-Hautea *et al.*, 1993b; Nodari *et al.*, 1993a; Vatokun *et al.*, 1993). Sets of RFLP probes obtained from different legume species have been used to study conservation of gene order (synteny) among legumes. Synteny was revealed among the related taxa of mungbean, cowpea, soybean and common bean (Menancio-Hautea *et al.*, 1993a; Boutin *et al.*, 1995). Common bean has, in comparison to other legume species, an intermediate genome size of 637 Mb (Arumuganathan and Earle, 1991). Consequently, a complete physical map of the common bean genome would provide an important mapping resource for several important legume species. Among legumes, common bean currently offers the only system in which CMS-related fertility restoration can be studied. Moreover, important genes for disease resistance (Haley *et al.*, 1993; Nodari *et al.*, 1993b; Yu *et al.*, 1993; Adam-Blondon *et al.*, 1994; Young *et al.*, 1998), seed proteins (Tague *et al.*, 1990; Hartweck *et al.*, 1997) and nitrogen fixation (Capote-Mainez and Sanchez, 1997) have been studied in common bean.

We have constructed the first large insert library in common bean, using a cv. Sprite snap bean derived genotype (Bassett and Shuh, 1982). We have used a modified BAC vector, into which a unique *EcoRI* cloning site had been introduced to create pECSBAC4 (Frijters *et al.*, 1997). This allows the combined use of restriction enzyme *EcoRI* and its cognate methylase in a competition assay (Larin *et al.*, 1991) to generate partially cleaved DNA for cloning. This method has

previously been reported to result in libraries with a relatively low proportion of clones carrying small inserts (Larin *et al.*, 1991; Bonnema *et al.*, 1996). In this paper we present a general characterization of the library and we report on the use of this library toward the positioning of the *Fr* restorer locus in bean.

## Materials and methods

### *High-molecular-weight (HMW) DNA preparation*

DNA from a Sprite snap bean-derived genotype (Bassett and Shuh, 1982) was used for library construction. Ca. 20 g of fresh or frozen leaf tissue was ground in liquid nitrogen using mortar and pestle. Purification of nuclei and embedding nuclei in low-melting (LM) agarose (Seaplaque, FMC) was performed according to Wing *et al.* (1993). Initial experiments indicated that less restriction enzyme was required and more reproducible digests were obtained with HMW DNA embedded in microbeads as compared to HMW DNA embedded in plugs. We, therefore, used microbeads for construction of the library. Care was taken to concentrate nuclei before they were embedded in microbeads, since DNA concentration in microbeads is usually lower than in plugs (Wang *et al.*, 1995).

### *Vector preparation*

A modified BAC vector, pECSBAC4 (Frijters *et al.*, 1997), was used for library construction. The pECSBAC4 vector was digested with *EcoRI* and cloned in pBluescript (Stratagene) to increase yield. The combined pBluescript/pECSBAC4 vector was grown overnight in 500 ml LB/chloramphenicol (25 µg/ml). Cells were harvested and DNA was prepared using the alkaline lysis method (Sambrook *et al.*, 1989). DNA was further purified on a resin column (Qiagen). A 3–5 µg portion of vector was digested simultaneously with *SacI* and *EcoRI*. The *EcoRI* digestion releases the pECSBAC4 vector from pBluescript, *SacI* digests within the polylinker of pBluescript only. After digestion, DNA was phenol/chloroform-purified and precipitated, and DNA ends were dephosphorylated using alkaline phosphatase (CIP, Boehringer Mannheim). The DNA was fractionated in a LM agarose gel (Seaplaque, FMC) to separate the pBluescript and pECSBAC4 vectors and to remove the short *SacI-EcoRI* polylinker fragment. Subsequently, pECSBAC4 was extracted from the gel and purified with GeneClean (Bio 101).

### *Partial digestion, CHEF gel electrophoresis*

Restriction enzyme *EcoRI* (New England Biolabs) and its cognate methylase, M.*EcoRI* (New England Biolabs), were used for partially digesting HMW DNA. Reactions were carried out in the presence of spermidine and BSA, as recommended by Creusot *et al.* (1992). After reactions were run to completion, beads were directly loaded onto a 1% LM gel (Seaplaque, FMC) and subjected to electrophoresis using a contour-clamped homogeneous electric field unit (CHEF DRII, BioRad) under the following conditions: 5 s initial and final switching time, 4 V/cm, 18 h running time, 0.5× TBE buffer. Under these conditions DNA fragments of 100 kb and larger were compressed as a single band.

### *Ligation*

After CHEF gel electrophoresis, a slice containing the partially digested HMW DNA was cut from the gel and the remainder of the gel was ethidium bromide-stained. Subsequently, the stained portion of the gel was used as a reference to cut away agarose pieces containing no DNA or fragments smaller than 100 kb from the unstained gel slice. The slice was treated with gelase (Epicenter) for 1 h at 45 °C to digest the agarose. Using a wide-bore tip, the DNA was pipetted on a membrane (Millipore) and dialyzed against 0.5× TE (Sambrook *et al.*, 1989) for 2–3 h at 4 °C. After dialysis, HMW DNA was ligated to vector using 400 units of ligase (New England Biolabs) overnight at 15 °C.

### *Transformation*

Prior to transformation, the ligation mixture was dialyzed for 3 h against 0.5× TE at 4 °C. ElectroMAX DH10B cells (13 µl, BRL) were added to 1 µl aliquots of ligation mix. Electroporation was carried out using an *E. coli* cell porater (BRL) at the low-voltage setting. After electroporation, cells were recovered in 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose pH 7.0) at 37 °C for 1 h. Cells were spun down, resuspended in 200 µl of SOC medium and plated on LB plates (Sambrook *et al.*, 1989) containing 25 µg/ml chloramphenicol, X-Gal and IPTG. After incubating for 36–48 h at 37 °C, white colonies were manually picked and stored in 384-well microtiter plates (Nunc

or Genetix), each well containing 60 µl of freeze-broth (36 mM K<sub>2</sub>HPO<sub>4</sub>, 13.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.7 mM sodium citrate, 0.4 mM MgSO<sub>4</sub>, 6.8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4.4% v/v glycerol, LB) and 25 µg/ml chloramphenicol. Bacteria were grown for 24 h and plates were stored at –80 °C.

### *Filter preparation and screening*

High-density filters were prepared by spotting clones onto Hybon N+ filters (Amersham) using a robot (Genetix). Filters were processed according to standard procedures (Sambrook *et al.*, 1989). DNA was fixed to membranes by UV crosslinking. Probes used in this study are listed in Table 1. RAPD markers were cloned in TA vectors according to the manufacturers' instructions (Invitrogen). Inserts were released from vector sequences by restriction digestion. Probes were radioactively labeled using the random primed method (Feinberg and Vogelstein, 1983). Hybridizations were done overnight at 65 °C in 0.5 M NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> pH 7.2, 7% SDS. Films (Fuji) were exposed for 1–5 days, depending on signal intensity.

### *BAC DNA manipulation*

BAC DNA was isolated according to Woo *et al.* (1994). To determine size, inserts were released using the rare cutter *NotI* (New England Biolabs). After digestion, a CHEF gel was run under the following conditions: 5 s initial switching time, 15 s final switching time, 6 V/cm, 17 h, 0.5× TBE. To assess the degree of overlap among individual BACs, DNA was digested with *EcoRI* and subjected to electrophoresis on a regular 0.8% agarose gel (BRL), 0.5× TBE.

## **Results**

### *Construction of the library*

It has been reported that incubating DNA with a restriction enzyme in combination with its cognate methylase results in a more reproducible partial digest than varying the amount of restriction enzyme or incubation time (Larin *et al.*, 1991; Bonnema *et al.*, 1996). Also, optimizing the restriction enzyme/methylase ratio increases the amount of fragments of the desired size range and, consequently, reduces the number of smaller fragments relative to time- or enzyme-limiting assays. These are important observations since both a high DNA concentration and elimination of small

Table 1. BAC clones identified after screening the library with various probes as indicated. Probes *ubc487* and *ubc528* are repetitive, the other probes are single-copy. Thirty-six clones were identified with an average insert size of about 100 kb.

Probe	Source	Number of clones	Size (kb)
<i>Phaseolin</i>	P. Gepts	5	75, 75, 80, 40, 115
<i>Bng 45</i>	Vallejos <i>et al.</i> (1992)	2	140, 130
<i>Bng 110</i>	Vallejos <i>et al.</i> (1992)	3	150, 150, 75
<i>Bng 64</i>	Vallejos <i>et al.</i> (1992)	2	100, 50
<i>Bng 143</i>	Vallejos <i>et al.</i> (1992)	3	60, 75, 100
<i>W2</i>	Vanhouten and Mackenzie, unpublished	5	130, 120, 70, 90, 100
<i>ubc487/ubc528</i>	He <i>et al.</i> (1995b); Vanhouten and Mackenzie, unpublished	12	120, 100, 150, 50 130, 120, 110, 80 150, 150, 90, 100
<i>AS-13</i>	J. Kelly	4	80, 75, 70, 120

fragments are crucial for the construction of a large insert library (Woo *et al.*, 1994). We used *EcoRI* and *EcoRI* methylase for partially digesting HMW DNA. Initial experiments established that a ratio of 120 units of methylase to 1 unit of restriction enzyme yielded most fragments in the desirable size range (200–400 kb). After resolution on a CHEF gel, these fragments were ligated to pECSBAC4 in a 100  $\mu$ l end volume. One  $\mu$ l of ligation mixture yielded, on average, ca. 100 white transformants. An earlier study indicated that electroporation at a lower voltage increases the relative number of recombinants with large inserts and decreases the fraction of blue colonies (Sheng *et al.*, 1995). We obtained both larger BAC clones and more white colonies per transformation at a lower voltage setting when compared to the medium voltage setting, consistent with results from another group (Frijters *et al.*, 1997). Over 33 000 clones were obtained from eight ligations and stored in microtiter plates at  $-80^{\circ}\text{C}$ .

#### Characterization of the library

To screen the library, high-density filters were prepared and hybridized to the various probes listed in Table 1. Each probe identified at least two BACs. To determine insert sizes, BAC plasmid DNA was digested with *NotI* and resolved on a CHEF gel, an example of which is presented in Figure 1. The average insert size of a total of 36 clones examined is close to 100 kb (Table 1).



Figure 1. CHEF gel electrophoresis of a subset of 22 selected BAC clones from Table 1. The ethidium bromide-stained gel displays clones after digestion with *NotI* and fractionated using the following conditions: 5 s initial switching time, 15 s final switching time, 6 V/cm, 17 h running time, 0.5 $\times$  TBE. Due to a low DNA concentration, bands are not visible in lanes 9 and 21.

Previously, a cosmid library had been constructed covering the entire mitochondrial genome (Janska and Mackenzie, 1993). To identify BAC clones potentially useful in extending mitochondrial DNA physical mapping studies, and to obtain an estimation of the level of contamination with organellar sequences, we probed the library with a set of mitochondrial cosmid clones representing the entire mitochondrial genome. Twenty-six hybridizing BACs were identified this way.

#### Hybridization with probes linked to *Fr*

CMS common bean can be restored to fertility by the action of nuclear restorer gene *Fr* (Mackenzie and Bassett, 1987; He *et al.*, 1995b). We have used molecular markers near *Fr* to develop a high-resolution map toward the positional cloning of this gene (He

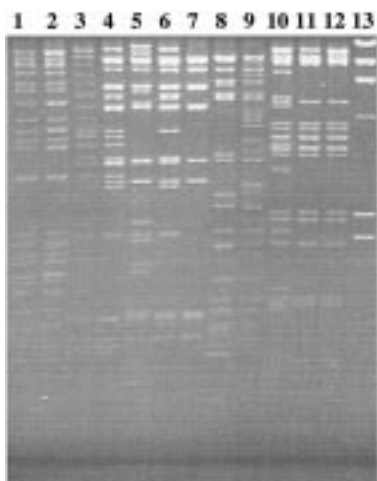


Figure 2. BAC clones hybridizing to both markers *ubc487* and *ubc528*. Clones were digested with *EcoRI* and resolved on a 0.8% regular agarose gel. Based on comigrating bands, these BACs can be grouped into a maximum of four contigs corresponding to lanes 1 and 2; lanes 3 through 7; lanes 8 and 9; lanes 10 through 12. Lane 13 contains a  $\lambda$  *HindIII*-digested molecular weight marker.

*et al.*, 1995b; and manuscript in preparation). Two flanking RAPD markers encompassing the *Fr* locus, *ubc487* and *ubc528*, were cloned and hybridized to total genomic DNA, revealing that these sequences correspond to medium repetitive DNA (not shown). Each probe identified over 400 clones after screening the library. Surprisingly, a number of BACs appeared to hybridize to both probes simultaneously (Table 1). When DNA from these cohybridizing clones was digested with *EcoRI* and resolved on an ethidium bromide-stained gel, they could be grouped into a maximum of four contigs based on overlaps established by comigrating fragments as shown in Figure 2. This result suggests that, given the genetic linkage between markers *ubc487* and *ubc528*, related families of repetitive DNA exist in the common bean genome that are probably linked to *Fr*.

## Discussion

### *Construction of the library*

We have constructed the first large insert library for common bean, using the modified BAC vector pECS-BAC4 and employing a *EcoRI*/M.*EcoRI* competition assay to generate partially cleaved DNA for cloning. Initially, we tried to partially cleave high-molecular-weight DNA by either limiting the amount of enzyme or incubation time. The major disadvantage to

both methods is the generation of small fragments that are difficult to eliminate via gel fractionation. Woo *et al.* (1994) used a second size selection to eliminate smaller 'trapped' DNA fragments from the size-selected gel slice. Although we obtained larger clones when applying a second size selection, the drop in transformation efficiency and a simultaneous increase in the number of false-positives rendered this method unsatisfactory in our hands. After probing the library with a set of probes covering the entire mitochondrial genome, we identified 26 candidate clones. This demonstrates that contamination of the library with mitochondrial sequences is very low, a result in agreement with results obtained by other groups when constructing a genomic library from purified nuclei (Wang *et al.*, 1995; Frijters *et al.*, 1997).

During the construction of the library, we routinely sampled BAC clones to determine the fraction of false-positives and insert size (data not shown). Based on these results, and on the results presented in Table 1, we estimated the average insert size of the library to be close to 100 kb. Assuming a haploid genome size of 637 Mb (Arumuganathan and Earle, 1991), this corresponds to a 4- to 5-fold coverage of the common bean genome. Hybridization with a number of single-copy probes generated, on average, 3.4 BAC clones per probe.

### *Screening the library with markers linked to Fr*

We have identified RAPD markers closely linked to *Fr* with the aim of constructing a contig encompassing this gene. A successful application of this strategy is facilitated when markers recognize low-copy or single-copy sequences. However, both markers *ubc487* and *ubc528* hybridized to repetitive DNA, and numerous BACs were identified with each probe after screening the library. Only twelve BACs appeared to hybridize to both probes simultaneously. Restriction analysis revealed that these BACs correspond to a maximum of four contigs. This result may have interesting implications regarding the use of multi-copy probes for the physical mapping of *Fr*. Markers *ubc487* and *ubc528* are genetically linked, and the identification of four contigs suggests physical linkage as well. These contigs failed, however, to amplify the polymorphic fragments that mapped these markers to each other and to *Fr*. Other, non-polymorphic bands could be amplified from these contigs, and specific contigs amplified specific bands (data not shown). We speculate several regions of repetitive DNA may

exist in the common bean genome, sharing a certain degree of homology to each other as revealed by the hybridization to *ucb487*, *ubc528*, or both. The observation that both markers are genetically linked to *Fr* raises the possibility for one or all of these contigs to be linked to *Fr* as well, demonstrating the potential for repetitive markers as tools for physical mapping. To verify this hypothesis we are now positioning the contigs genetically.

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