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Construction of a bacterial artificial chromosome library containing large *Eco*RI and *Hin*dIII genomic fragments of lettuce

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Abstract Existing bacterial artificial chromosome (BAC) vectors were modified to have unique *Eco*RI cloning sites. This provided an additional site for generating representative libraries from genomic DNA digested with a variety of enzymes. A BAC library of lettuce was constructed following the partial digestion of genomic DNA with *Hin*dIII or *Eco*RI. Several experimental parameters were investigated and optimized. The BAC library of over 50,000 clones, representing one to two genome equivalents, was constructed from six ligations; average insert sizes for each ligation varied between 92.5 and 142 kb with a combined average insert size of 111 kb. The library was screened with markers linked to disease resistance genes; this identified 134 BAC clones from four regions containing resistance genes. Hybridization with low-copy genomic sequences linked to resistance genes detected fewer clones than expected from previous estimates of genome size. The lack of hybridization to chloroplast and mitochondrial sequences demonstrated that the library was predominantly composed of nuclear DNA. The unique *Eco*RI site in the BAC vector should allow the integration of BAC cloning with other technologies that utilize *Eco*RI digestion, such as AFLP™ markers and *Rec*A-assisted restriction endonuclease (RARE) cleavage, to clone specific large *Eco*RI fragments from genomic DNA.

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Introduction

Map-based cloning is becoming increasingly efficient in plants (Tanksley et al. 1995). Disease resistance genes and several other types of genes have been recently isolated from tomato and *Arabidopsis* by map-based cloning, demonstrating the power of this approach (Arondel et al. 1992; Bent et al. 1994; Giraudat et al. 1992; Grant et al. 1995; Martin et al. 1993; Mindrinos et al. 1994; Song et al. 1995; Dixon et al. 1996). Most of these studies utilized yeast artificial chromosome (YAC) libraries of genomic DNA (Burke et al. 1987). However, YAC libraries are time-consuming to generate and to manipulate. A current rate-limiting step for routine map-based cloning from crop species is the efficient generation of libraries containing large inserts of genomic DNA.

Bacterial artificial chromosome (BAC) vectors have recently been developed from the mini-F plasmid pMBO131 (O'Connor et al. 1989) that allow cloning and stable maintenance of large DNA fragments in *E. coli* (Shizuya et al. 1992). BAC libraries have several advantages over YAC libraries. Several of the problems encountered with YAC libraries such as low transformation efficiencies or co-transformation (Bellis et al. 1991; Wada et al. 1990), unstable clones (Dunford et al. 1993; Neil et al. 1990; Schmidt et al. 1994), chimerism (Green et al. 1991; Libert et al. 1993; Schmidt et al. 1994), occur less often with BAC libraries. The transformation efficiencies of BACs into *E. coli* are at least 100 times higher than the transformation of YACs into yeast (Shizuya et al. 1992; Wang et al. 1995; Woo et al. 1994). BAC vectors contain the replication and partition sequences *par*A and *par*B from the F(ertility)-factor that ensure low-copy maintenance of plasmid DNA and prevent two BACs being maintained in a single cell (Willetts and Skurray 1987); these sequences reduce

instability and problems resulting from co-transformation. The limited number of BAC clones studied to date have been stable and non-chimeric (Jiang et al. 1995; Shizuya et al. 1992; Wang et al. 1995; Woo et al. 1994). Furthermore, *E. coli* grows about five times faster than yeast and, in contrast to YACs, BACs can be easily isolated as supercoiled plasmids using standard miniprep procedures, thereby greatly facilitating construction and characterization of BACs. BAC libraries have now been reported for two monocot plants, sorghum and rice, with average insert sizes of 157 and 125 kb, respectively (Wang et al. 1995; Woo et al. 1994; Zhang et al. 1996). The sizes of these BAC libraries are nearly the same as those for several of the earlier plant YAC libraries (Edwards et al. 1992; Grill and Somerville 1991; Martin et al. 1992). However, the maximum size obtained with YAC clones (1+ Mb) exceeds that so far achieved for BAC clones (300+ kb). Therefore, if large inserts are required, YAC libraries are still necessary.

Previous BAC vectors had unique *Hin*dIII, *Bam*HI and *SphI* sites available for cloning. The BAC libraries of sorghum and rice utilized the unique *Hin*dIII restriction site (Wang et al. 1995; Woo et al. 1994; Zhang et al. 1996). BAC vectors with a unique *Eco*RI site for cloning would be useful for several reasons. *Eco*RI is a reliable endonuclease that has been used to generate several YAC libraries (Edwards et al. 1992; Libert et al. 1993; Martin et al. 1992). Moreover, the use of this restriction enzyme in combination with *Eco*RI methylase provides reproducible partial digestions (Larin et al. 1991). A unique *Eco*RI site would also make the BAC system compatible with other techniques such as *RecA*-assisted restriction endonuclease (RARE) cleavage (Ferrin and Camerini-Otero 1994) and amplified fragment length polymorphism (AFLP™) markers (Vos et al. 1995).

We are isolating several genes for disease resistance from lettuce using map-based cloning (Michelmore et al. 1994). In lettuce, as in other plant species, disease resistance genes are organized in large clusters. We are developing physical contigs of these regions to study the organization and evolution of these clusters. We have therefore generated a BAC library and identified large genomic fragments from these regions. During the construction of the library we optimized several experimental variables. In addition, we modified existing BAC vectors to provide a unique *Eco*RI site for cloning. The library was made following partial digestion with either *Hin*dIII or *Eco*RI. It was evaluated for the presence of single-copy and multi-copy nuclear sequences as well as for insert stability and contamination with organellar sequences.

Materials and methods

Construction of BAC vectors with a unique *Eco*RI cloning site

pECBAC1 was derived from pBELOBAC11, kindly provided by Drs. H. Shizuya and M. Simon (California Institute of Technology, Cal.f.) by the ligation of three fragments. The vector pBELOBAC11 was digested with *Nco*I and *Eco*RI to provide a 5.2-kb fragment containing the majority of the vector. A 1.9-kb fragment containing the *lacZ* region and the multiple cloning site was obtained by digestion of pBELOBAC11 with *Nco*I. Finally, a 426-bp fragment containing the majority of the chloramphenicol (Cm) resistance gene was amplified by polymerase chain reaction (PCR) from pBELOBAC11. Primers were designed to introduce an *Apo*I site in place of the *Eco*RI site in the Cm resistance gene: 5′-CGCCTGAT-*GAATTTA*CATCTGGAATTA-3′ (italics: *Apo*I site, underlined: sites of directed mutagenesis) and 5′-CATTAAGCATTCTG-CCGACATGGAAGC-3′. The amplified product was cut with *Apo*I and *Nco*I to yield a 310-bp fragment. The 5.2-, 1.9- and 0.31-kb fragments were ligated together. The resulting plasmid had only one *Eco*RI site remaining (within the *lac*Z gene) due to the fusion of the *Apo*I and *Eco*RI ends within the Cm resistance gene and was named pECBAC1.

A spectinomycin (Spec) resistance gene was introduced into pECBAC1 from pBELOBACSPEC (unpublished; kindly provided by A. Lloyd, Stanford University) to generate pECSBAC4. A 2.3-kb *Hin*dIII – *Sca*I fragment from pBELOBACSPEC was ligated to a 7-kb *Hin*dIII – *Sca*I fragment of pECBAC1. Both vectors have unique *Hin*dIII and *Sca*I sites. Both vectors were transformed by electroporation into *E. coli* electroMAX DH10B cells (Gibco BRL).

Preparation of BAC vector

BAC vectors pBELOBAC11 and pECSBAC4 were isolated by procedures described previously (Wang et al. 1995; Woo et al. 1994). Cells were harvested from 4 of overnight culture in LB medium containing 12.5 μg Cm (O.D. = 1.4). Plasmid DNA was isolated using the Plasmid Maxi Kit (Qiagen) followed by cesium chloride density gradient centrifugation. The BAC vectors were digested to completion with either *Hin*dIII or *Eco*RI, followed by dephosphorylation with HK phosphatase (Epicenter Technologies). The efficiency of dephosphorylation was tested by self-ligation of the BAC vector compared to self-ligation of non-dephosphorylated BAC vector; only preparations in which dephosphorylation resulted in more than a 98% reduction in the number of transformants were used for construction of the BAC library. The cloning efficiency of each vector preparation was tested by ligation with genomic DNA that had been completely digested with *Hin*dIII or *Eco*RI; only those preparations that resulted in a minimum of 50% white colonies were used for library construction.

Preparation of plant high-molecular-weight (HMW) DNA

Plants of *Lactuca sativa* cv 'Diana' (*Dm1*, *Dm3*, *Dm7* and *Dm8*) were grown in a growth-chamber for 6–8 weeks. Nuclei were isolated from leaves using a nuclei isolation buffer (Zhang et al. 1995) optimized for lettuce (0.35 *M* sorbitol; 0.1 *M* TRIS-base; 5 m*M* EDTA; 20 m*M* Nabisulfate, pH 7.5), resuspended in SCE buffer (1 *M* sorbitol; 0.1 *M* Na citrate; 60 m*M* EDTA, pH 7.0) and embedded in low-melting point agarose plugs. Plugs were incubated in 0.5 *M* EDTA (pH 9.0), 1% (w/v) sodium lauryl sarcosine and 0.2 mg/ml proteinase K (Boehringer Mannheim) at 50°C for 2 days with one change of buffer and stored at 4°C. Prior to enzyme digestion, plugs were dialysed overnight against TE buffer plus 40 μg/ml PMSF (phenyl methyl sulphonyl fluoride) at 4°C. The concentration of PMSF was increased to 80 μg/ml followed by an additional incubation for 1 h at room temperature to ensure complete inactivation of proteinase K. Prior to partial digestion, plugs were washed four times in TE for 30 min at room temperature.

For partial digestions using *Hin*dIII, the plugs were equilibrated twice with *Hin*dIII restriction buffer (50 m*M* NaCl, 10 m*M* TRIS-HCl, 10 m*M* $MgCl_2$ and 1 m*M* dithiothreitol, pH 7.9) for 1 h at

room temperature. Plugs were melted at 65°C for 15 min and held at 37°C for 5 min. For each plug (75 μl), containing approximately 15 μg DNA, 7 U *Hin*dIII (New England Biolabs) was added and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1/10 volume of 0.5 M EDTA, pH 8.0.

For partial digestions using *Eco*RI and *Eco*RI methylase, the plugs were equilibrated twice in *Eco*RI/*Eco*RI methylase buffer [100 m*M* NaCl, 100 m*M* TRIS-HCl (pH 7.5), 2 m*M* $MgCl_2$, 80 μ*M* S-adenosylmethionine (New England Biolabs), 0.5 mg/ml bovine serum albumin, 2.6 m*M* spermidine trihydrochloride, and 1 m*M* dithiotheitol] for 30 min. Each plug was then cut in six equal pieces (approximately 12.5 μl) and placed in 1.5-ml Eppendorf tubes on ice. Twenty units *Eco*RI and 40–50 U *Eco*RI methylase (both NEB) were added to each tube with 67.5 μl of the *Eco*RI/*Eco*RI methylase buffer and equilibrated for 1 h on ice before transfer to 37°C for 4.5–5.0 h. The reaction was terminated by the addition of EDTA (20 m*M* final conc.) and proteinase K (0.5mg/ml final conc.) and incubation at 37°C for 30 min. Alternatively, partial digestion without *Eco*RI methylase was performed using the same procedure except that DNA was digested with *Eco*RI alone for 1 h at 37°C.

Large fragments of genomic DNA were selected by two rounds of size fractionation using pulsed field gel electrophoresis (PFGE). In the first size fractionation, DNA fragments were separated in a 1% SeaKem agarose gel (FMC, Rockland, Me) in 0.5 TBE (45 m*M* TRIS, 45 m*M* TRIS-borate, 1 m*M* EDTA). PFGE was conducted for 16 h at 14°C to generate a compression zone in excess of 1 Mb using a contour clamped homogeneous electric field (CHEF) DRIII apparatus (BioRad). The region of the gel containing 200-to 450-kb fragments was excised and directly used for the second size fractionation in a 0.8% SeaPlaque (low-melting-point) agarose (FMC) using conditions that focused DNA larger than 200 kb into a tight band. Prior to ligation, each 100 mg of gel was incubated with Gelase (Epicenter Technologies). Each 100-μl fraction containing approximately 60 ng lettuce DNA was directly used for ligation with the BAC vector.

Construction of the BAC library

Each 100-μl fraction containing large *Hin*dIII genomic fragments was ligated with 20 ng of BAC vector without dilution. The fractions containing large *Eco*RI DNA fragments were used at 3 × and 10 × dilutions as well as undiluted. Ligations with each fraction were performed in 1 × ligase buffer with 500 U T4 DNA ligase in (NEB) and overnight incubation at 16°C. Enzymes were heat-inactivated for 10 min at 65°C. Ligations were stored at 4°C until used. If salts were removed prior to electroporation, the ligation product was dialysed for 3 days at 4°C using a Ultrafree-MC filter (30,000 NMWL) with a polysulfone membrane (Millipore, Bedford, Mass.) in a 1.5-ml Eppendorf tube with three changes of TE buffer every 24 h.

Each 0.5 μl of ligation product was transformed into 13 μl of electrocompetent ElectroMAX DH10B cells (Gibco BRL) using a Cell-Porator system (Gibco BRL) following the manufacturers protocol. White recombinant colonies were picked directly to 384-well plates (Nunc) containing LB freezing buffer [LB, 12.5 μg/ml Cm, 36 m*M* K_2HPO_4, 13.2 m*M* KH_2PO_4, 1.7 m*M* Na citrate, 0.4 m*M* $MgSO_4$, 6.8 m*M* $(NH_4)_2SO_4$, 4.4% (v/v) glycerol].

Supercoiled BAC plasmid was isolated from 1.5-ml cultures grown overnight in LB with 12.5 μg/ml Cm using an alkaline lysis procedure (Sambrook et al. 1989). DNA pellets were dissolved in 25 μl TE. Insert size was determined by digestion of half the sample with *Not*I (Gibco BRL) and fractionation using PFGE.

Screening of the BAC library

For screening by hybridization, high-density filters were made by replication of four 384-well plates onto sterile Hybond N + filters (8.5 × 11.5 cm) (Amersham) that had been prewetted on LB agar containing 12.5 μg/mlCm, using a BIOMEK 1000 robot (Beckman). Filters were incubated overnight on LB medium with *Cm*. Colonies were lysed on the membrane (Nizetic et al. 1990) and hybridized to random hexamer [^{32}P]-labeled DNA probes (Feinberg and Vogelstein 1984).

Screening by PCR was performed using sequence characterized amplified region (SCAR) primers (Paran and Michelmore 1993) on super-pools and pools of DNA of 2,304 and 384 BAC clones, respectively. Each plate in the library was replicated to a 384-well plate containing LB and 12.5 μg Cm. After overnight incubation, cells were harvested and pooled from each plate. Plasmid DNA was isolated by an alkaline lysis procedure (Sambrook et al. 1989) in microfuge tubes. Superpools were constituted from DNA of six 384-well plates, each with approximately the same DNA concentration.

DNA probes

Total genomic DNA from cv 'Diana' was extracted from leaves using a modified CTAB protocol (Murray and Thompson 1980; Bernatzky and Tanksley 1986; Landry et al. 1987). The probes for chloroplast (cp) and mitochondrial (mt) sequences were kindly provided by J. Palmer (University of Indiana). These consisted of seven clones of lettuce cpDNA covering 79 kb as well as mitochondrial sequences from carrot (*ND5* exon 2), petunia (*ND1* exon E), tobacco (*atp9*) and *Oenothora* (*atpa* and *cob*). Clones were labeled individually and then pooled to generate two probes, one for chloroplast and the other for mitochondrial sequences. Probes CL922, CL1419, CL207, CL1795 and CL877 are restriction fragment length polymorphism (RFLP) markers linked to clusters of *Dm* genes that were derived from random lettuce cDNA clones (Landry et al. 1987). I11, AC15 and K13 are cloned fragments of random amplified polymorphic DNA (RAPD) markers tightly linked to *Dm3* (Anderson et al. 1996; Okubara et al. 1994). IPCR36 is a sequence flanking a T-DNA insertion close to *Dm3* (Okubara and Michelmore, unpublished). Probe CL1618 is potentially linked to the recessive gene, *mo*, for resistance to lettuce mosaic virus (Irwin and Michelmore, unpublished).

Results

Construction of BAC vectors with a unique *Eco*RI cloning site

The BAC vectors pBELOBAC11 and pBELOBAC-SPEC contain two *Eco*RI sites, one in the polylinker of the *β*-galactosidase gene and one in the chloramphenicol resistance gene. The *Eco*RI site of the Cm gene of pBELOBAC11 was destroyed by site-directed mutagenesis using PCR as described in the Materials and methods. One chloramphenicol-resistant transformant was selected from the tri-molecular ligation. The plasmid in this transformant, pECBAC1, produced a single linear 7.4-kb fragment when cut with *Eco*RI which was the same size as pBELOBAC11 cut with *Hin*dIII. A spectinomycin resistance gene was transferred from pBELOBACSPEC into pECBAC1 to produce pECSBAC4 (Fig. 1). Because the fragment with the spectinomycin resistance gene contains a *Sph*I site, the *Sph*I site in the multiple cloning site in pECSBAC4 is no longer unique. The primer used for site-directed

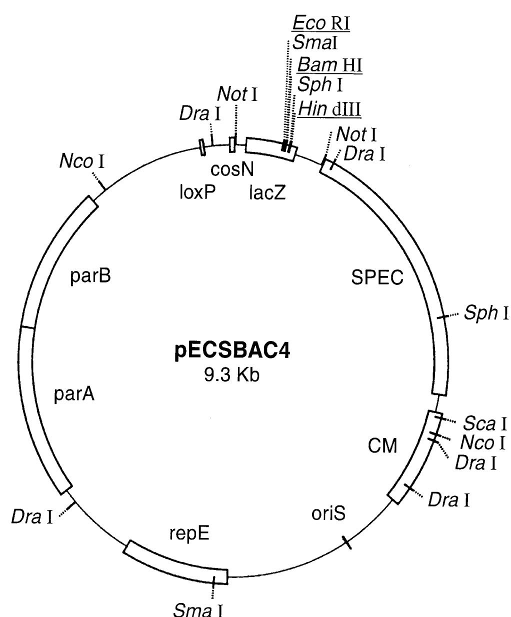

Fig. 1 BAC vector pECSBAC4 that was derived from pBELOBAC11. *CM* chloramphenicol resistance gene, *SPEC* spectinomycin resistance gene, *par*A and *par*B partition loci ensuring maintenance at single copy, *ori*S and *rep*E unidirectional origin of replication, *cos*N bacteriophage λ cleavage site, *lox*P site for P1 *Cre*-mediated recombination. Three unique cloning sites (*underlined*) are available in *lac*Z: *Eco*RI, *Bam*HI and *Hin*dIII

mutagenesis maintained the open reading frame of the Cm gene and changed two amino acids in the gene product. The level of Cm resistance was tested by comparing the growth of transformants with pECSBAC4 and pBELOBAC11 in liquid cultures with a range of concentrations of Cm. Parallel growth-curves were obtained with 12.5, 25 or 75 μg/ml Cm; 75 μg/ml Cm resulted slightly delayed growth for both cultures compared to lower concentrations. Therefore, the introduced changes had no detectable effect on chloramphenicol resistance.

Construction of a BAC library of *Lactuca sativa*

A BAC library was constructed using six ligations. During the construction of this library several experimental parameters were optimized as described below. Initially, while pECSBAC4 was being constructed, genomic DNA was partially digested with *Hin*dIII, and two ligations were made using pBELOBAC11 as the vector. Subsequently, HMW DNA was partially digested either with *Eco*RI alone (one ligation) or with *Eco*RI in combination with *Eco*RI methylase (three ligations) and ligated into pECSBAC4. The six ligations resulted in a total of nearly 53,000 white colonies. The insert sizes were determined after each ligation by digestion of randomly selected white colonies with *Not*I followed by PFGE. Hybridization of clones to total genomic DNA demonstrated that the inserts were of lettuce origin (Fig. 2). The frequency of clones with inserts ranged from 81% to 100% for the different ligations. The average insert size per ligation varied between 92.5 and 142 kb. The average insert size from a ligation was negatively correlated with the frequency of white colonies. Of the clones tested, approximately 14% of the inserts in the *Hin*dIII library and 25% of the inserts in the *Eco*RI library contained internal *Not*I sites as evidenced by multiple fragments (Fig. 2). This is less frequent than observed in BAC libraries of sorghum and rice (Wang et al. 1995; Woo et al. 1994). Currently, the library consists of approximately 50,000 clones with inserts of lettuce DNA comprising a total of 5,500 Mb (Table 1). Assuming a haploid genome size of $2.3–2.7 \times 10^9$ bp for lettuce (Arumuganathan and Earle 1991; Michaelson et al. 1991; Galbraith and Michelmore, unpublished), the library represents approximately two genome equivalents.

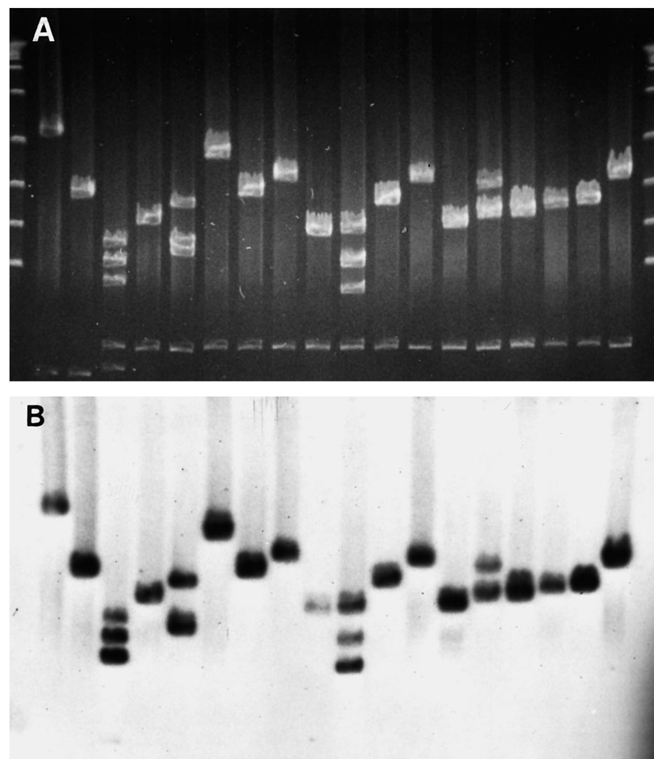

Fig. 2A, B Analysis of lettuce BAC clones. DNA was isolated from 18 randomly selected BAC clones by an alkali miniprep procedure followed by digestion with *Not*I. The *first two lanes* contain fragments cloned into pBELOBAC11; the *remaining 16 lanes* contain fragments cloned into pECSBAC4. **A** Ethidium bromide-stained gel containing the digested DNA fragments after separation by PFGE. Size markers in the *outer lanes* are concatemers of λ phage (48.5 kb). **B** Southern blot of this gel showing hybridization of BAC inserts to radioactively-labeled total genomic DNA of *Lactuca sativa*. *Lanes 3, 5, 10 and 14* contain four, two, three, and two genomic fragments, respectively, due to internal *Not*I sites

Table 1 Characteristics of the lettuce BAC library determined from randomly selected clones derived from six different ligations

Ligation number	Restriction enzyme used for partial digests	Number of white colonies per microliter ligation product	Total no. of white colonies picked	Number without insert/no. white colonies tested (%)	Average insert size[a] (kb)	Mb cloned
1	*Hin*dIII	238	19,000	0/30 (0%)	92.5	1,758
2	*Hin*dIII	63	4,000	2/34 (5.9%)	112.5	423
3	*Eco*RI	125	9,990	1/36 (3.8%)	112.5	1,081
4	*Eco*RI[b]	86	8,270	1/26 (3.8%)	117	931
5	*Eco*RI	108	7,550	8/43 (18.6%)	142	873
6	*Eco*RI	54	4,180	5/42 (11.9%)	138.5	519
Total			52,990		111	5,576

[a] Colonies without inserts were not included when calculating the average insert size
[b] Partial digestion performed without *Eco*RI methylase

Partial endonuclease digestion

High-molecular-weight DNA was isolated from nuclei of *L. sativa* cv 'Diana' (*Dm1*, *Dm3*, *Dm7* and *Dm8*). Digestion with 7 U *Hin*dIII at 37°C for 30 min yielded the most DNA, between 200 and 450 kb per melted 75-μl plug that contained approximately 15 μg DNA. To minimize shearing of the HMW DNA, we performed partial digestions with *Eco*RI and *Eco*RI methylase with intact plugs. As a consequence much more restriction enzyme and longer incubation times were required. Incubation of one-sixth of a 75 μl plug with 20 U *Eco*RI and 40–50 U *Eco*RI methylase for 5 h at 37°C resulted in the same degree of partial digestion as the completely melted plug digested with *Hin*dIII as above. A similar degree of digestion was obtained with 20 U *Eco*RI without *Eco*RI methylase and 1 h of incubation.

Optimization of conditions

We investigated both the number of size fractionations required and the optimal size range. When DNA was fractionated using only one round of PFGE, DNA from the 200 and 450 kb region of the gel resulted in BAC clones with average insert sizes between 50 and 75 kb. The small size was probably caused by smaller DNA fragments being entrapped in the larger molecules, resulting in small plasmids that transformed with greater efficiency. Therefore, two rounds of size selection, the first to allow selection of 200- to 450-kb fragments and a second to concentrate the large fragments into a single compression zone and to remove small molecules trapped in the first round, were used to provide clones with average insert sizes of over 100 kb.

The maximum size of HMW DNA that could be reliably cloned was detemined by investigating three different ranges for the first size selection. 100–300 kb, 200–450 kb and 400–800 kb. The isolated agarose pieces were directly subjected to a second size selection using PFGE conditions to create a compression zone starting at 100, 150 and 350 kb respectively. Average insert sizes for the 100- to 300-kb and 200- to 450-kb fractions were 60 and 117 kb respectively. The 400- to 800-kb fraction resulted in very few white transformants; of these 75% contained no inserts and the remainder were smaller than 50 kb. Therefore, fragments of 200–450 kb were used in the first selections of subsequent experiments.

To determine the optimum concentration of lettuce DNA to be ligated to the vector, we tested a variety of dilutions. Different dilutions of the Gelase-treated blocks (approximately 60 ng DNA: undiluted, 3 × and 10 × diluted) were ligated with 20 ng vector in a 100 μl reaction volume. The 3 × and 10 × diluted blocks resulted in 2- to 11-fold and 0- to 2-fold, respectively, more white colonies than the undiluted blocks. The concentration of the vector was also varied in one experiment to determine the optimal concentration. The use of more or less than 20 ng of BAC vector in the ligation reduced the number of white colonies per microliter ligation. Therefore, for the last three ligations, 20 ng of vector was routinely ligated to blocks that had been diluted 3 ×.

A range of field strengths was tested to determine the optimum electroporation conditions using DNA from ligation 3 (Table 1). Electroporation of *E. coli* strain DH10B electro-competent cells (Gibco BRL) at 14 kV/cm resulted in a higher number of colonies per microliter ligation than the recommended settings of the Cel-porator system (16.5 kV/cm) (Fig. 3). The effect of removing salts by dialysis was also tested. Dialysis increased the number of transformants per microliter at most of the field strengths tested (Fig. 3). Therefore, the remainder of ligation 3 and ligations 4, 5 and 6 were dialysed and electroporation performed using a field strength of 14 kV/cm.

Characterization of the BAC library

The frequency of clones containing organellar DNA was determined by hybridizing filters containing

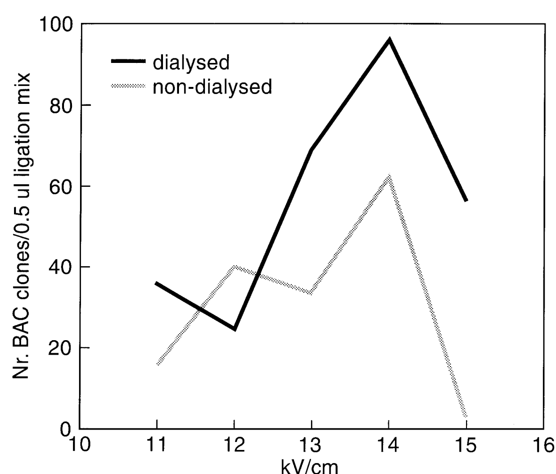

Fig. 3 The relationship between field strength during electroporation and the number of transformants obtained. A 0.5-μl aliquot of ligation product was added to 13 μl of electroMAX DH10B cells (Gibco BRL) and placed between 0.15-cm gap electrodes. Different field strengths were tested with a constant pulse time of 6 ms. The settings for the Cell-porator system (Gibco BRL) were 270–400 V; 330 μF capacitor; low resistance and fast charge rate with the 4,000 ohms resistor on the voltage booster unit

4×384 BACs/filter to seven chloroplast clones from lettuce and six mitochondrial sequences from other plant species (see Materials and methods). DNA from these clones was included as positive and negative controls. No hybridization of BAC clones was detected with either the chloroplast or mitochondrial probes (data not shown). This confirmed that the extraction procedure effectively purified nuclei and that the library is comprised predominantly of nuclear sequences.

Screening the library with markers linked to downy mildew resistance genes

The library was made from cv 'Diana' which contains four genes for resistance to downy mildew (*Dm1*, *Dm3*, *Dm7* and *Dm8*) as well as the dominant allele for susceptibility to lettuce mosaic virus (*Mo*). We screened the library by hybridization and by PCR. Ten probes that detect single-copy sequences or small multigene families clustered close to resistance genes (Anderson et al. 1996; Landry et al. 1987) were hybridized to libraries containing *Hin*dIII and *Eco*RI genomic fragments (Table 2). The three single-copy probes detected 0–5 BAC clones. The three two-copy probes detected 1 or 2 clones. The number of clones detected with these low-copy probes was half that predicted based on previous estimates of genome size for lettuce (Arumuganathan and Earle 1991; Michaelson et al. 1991; Galbraith and Michelmore unpublished). As expected, multiple clones containing the AC15, K13 and CL1795 sequences were identified; the precise copy number of these sequences is unknown. The mean size of clones identified by markers linked to resistance genes was 27% larger that the average estimated from random clones. The selected BAC clones represent a significant amount of the major clusters of resistance genes in lettuce.

Table 2 BAC clones identified by probes linked to disease resistance genes

Marker	Linkage[a]	Genomic copy no.[b]	Number of clones identified			Mean insert size in kb (no. of clones)[c]
			*Hin*dIII library	*Eco*RI library	Total	
CL922	*Dm1/3*	1	0	0	0	
CL1419	*Dm1/3*	2	1	1	2	130 (2)
I11	*Dm1/3*	2	1	1	2	210 (2)
IPCR36	*Dm1/3*	5	3	1	4	86 (4)
AC15	*Dm1/3*	10 +	13	23	36	140 (36)
K13	*Dm1/3*	20 +	17	21	38	155 (12)
CL207	*Dm4/7*	1	4	1	5	124 (5)
CL877	*Dm5/8*	2*	0	1	1	150 (1)
CL1795	*Dm1/3* *Dm4/7* *Dm5/8*	15 +	20	25	45	148 (8)
CL1618	*mo*	1	0	1	1	130 (1)
Total			59	75	134	142 (55)

[a] The *Dm1/3*, *Dm4/7* and *Dm5/8* clusters include at least 11, 4, and 6 genes, respectively, for resistance to five different pathogens (Michelmore et al. 1994)

[b] As estimated by the minimum number of fragments detected in genomic Southerns following digestion with each of several endonucleases with the exception of *IPCR36* that was determined by densitometry of an insertion mutant (Okubara and Michelmore, unpublished). (* Both copies of *CL877* may be on this BAC)

[c] Some clones hybridized to multiple probes. Such clones were only included once in the calculation of average insert size

The BAC library containing large *Hin*dIII fragments was also screened by PCR using SCAR markers for AC15 and IPCR36. The PCR was performed first on superpools of 2,304 clones (six 384-well plates). When a PCR product of the expected size was detected, pools of BAC clones from the constituent individual 384-well plates were screened. For AC15, twelve 384-well plates were detected which produced a PCR product; in 9 cases, the expected 800-bp PCR product was detected. Some of the reactions contained smaller fragments; these fragments were also detected in the PCR of genomic DNA performed as a control. For IPCR36, two 384-well plates contained clones with the expected 660-bp fragment. All 9 AC15-containing and 2 out of 3 IPCR36-containing BAC clones that were detected by hybridization to *Hin*dIII fragments were also localized to plates by PCR. PCR detected 3 plates with AC15-containing BAC clones that were not detected by hybridization. Although PCR rapidly identified plates containing positive clones, it would have been considerable work to identify individual colonies. Once the filters had been generated, hybridization was not significantly slower and allowed the rapid identification of individual clones. Therefore, hybridization was used to select genomic clones.

Discussion

The lettuce BAC library

We have generated a one-to two-genome equivalent BAC library of lettuce using partial *Hin*dIII and *Eco*RI digests. Two enzymes were used to reduce the chances of cloning bias due to non-uniform distribution of restriction sites. The frequencies with which positive clones for a variety of probes were detected were similar for both libraries (Table 2), although the low numbers of clones identified would not have revealed specific cloning biases. There is a strong negative correlation between the number of colonies obtained and the average insert size (see below). In order to obtain sufficient numbers of clones, we had to compromise slightly on the average insert size. The lettuce BAC library consists of approximately 50,000 clones with an average insert size of 111 kb comprising a total of 5,500 Mb of nuclear DNA. Although this insert size is slightly smaller than other BAC (Wang et al. 1995; Woo et al. 1994) and YAC libraries (Edwards et al. 1992; Grill and Somerville 1991; Martin et al. 1992) consisting of fewer clones for other plant species, it did allow us to isolate numerous clones from regions of interest, even though lettuce has a genome size greater than those of most of these other source species. The genome coverage indicated by the number of clones selected from the library was approximately half that expected from five previous estimates of haploid genome size based on measurements of whole stained nuclei (2,300–2,700 Mb) (Arumuganathan and Earle 1991; Michaelson et al. 1991). This inconsistency may be due to an under-estimation of genome size, an over-estimation of the copy number of some of the low-copy probes, or under-representation of sequences from resistance gene regions in these libraries.

We were able to isolate numerous clones from genomic regions containing disease resistance genes. The chromosomal regions containing disease resistance genes contain a mixture of low-copy and high copy repeated sequences with extensive duplication (Anderson et al. 1996). We were able to detect clones for most probes. The average size of the clones detected with specific probes was larger than the size of the random clones sampled after each ligation (Table 2). These are currently being assembled into contigs of overlapping clones (Meyers and Michelmore, unpublished). The percentage of chimeric clones in our BAC library has not been studied. However, chimerism was not detected in BAC libraries of sorghum (Woo et al. 1994) and rice (Jiang et al. 1995) when investigated by FISH (fluorescence in situ hybridization). In the limited contig analysis performed so far, we have not detected any chimeric clones. We have also not detected instability in the BAC clones from these regions either in formal investigations of stability over serial subcultures or during characterization spanning a year. This is consistent with the stability of BAC clones of human, sorghum and rice sequences (Shizuya et al. 1992; Wang et al. 1995; Woo et al. 1994).

Critical factors in the construction of BAC libraries

The quantity and quality of input genomic DNA are two of the most critical factors. The BAC cloning protocol requires a series of compromises. While the preparation of HMW DNA from isolated nuclei resulted in significant shearing; it resulted in only a very low frequency of contaminating organellar sequences. Sufficient DNA is required to compensate for the lower transformation efficiencies of large plasmids (Leonardo and Sedivy 1990; Sheng et al. 1995; Wang et al. 1995); nevertheless, if the pulsed field gels are overloaded, there will be excessive trapping of smaller DNA fragments which then co-migrate with larger DNA fragments on a PFG. These smaller molecules transform with much greater efficiency, and the average insert size drops dramatically. A significant amount of these smaller fragments can be excluded by a second size fractionation; this results in an increase in the average insert size of BACs, but a drop in the number of clones due the decrease in the amount of DNA.

The average insert size of BACs for a given ligation was negatively correlated with the number of BACs generated per microliter of input DNA, as has been observed earlier (Leonardo and Sedivy 1990; Woo et al. 1994). Our first ligation had an average insert size of

93 kb and resulted in 19,000 BACs, whereas only 4,180 BACs were isolated in the sixth ligation with an average insert size of 139 kb. Greater amounts of the genome were cloned by tolerating a slightly lower insert size. The small gains in average insert size were offset by big decreases in cloning efficiency. Although the genomic DNA was selected twice to be at least 200 kb, the average size of all ligations was well below this. This has been consistently observed during the construction of other BAC libraries (Wang et al. 1995; Woo et al. 1994) and suggests that the majority of molecules cloned are trapped among larger molecules and that if sizing could be done more effectively, there would be a concomitant increase in efficiency. The range of fragment sizes generated by partial digestion would also influence the efficiency of cloning. We detected no obvious difference in the sizes of inserts or in the cloning efficiency following partial digestion with *Hin*dIII or *Eco*RI with and without competition with *Eco*RI methylase. Selection of DNA fragments larger than 400 kb did not result in clonable large fragments. The largest BAC clone so far isolated from lettuce was approximately 350 kb. This is similar to the largest clones in other BAC libraries (Shizuya et al. 1992; Wang et al. 1995; Woo et al. 1994) and is therefore probably close to the maximum size of BACs obtainable with the current BAC technology.

Optimal concentrations of insert and vector are also the result of a compromise between conflicting requirements. The ligation of large genomic fragments to a BAC vector is similar to ligations for the construction of jumping libraries. For both, large genomic fragments have to be circularized with much smaller plasmids. The importance of diluting the large fragments sufficiently to reduce the likelihood of intermolecular ligation has been demonstrated for jumping libraries (Collins and Weismann 1984; Poustka and Lehrach 1988). The larger the fragments to be cloned, the more they should be diluted. In addition, for the ligation of a small vector molecule to one end of the long DNA fragment followed by ring closure, the concentration of the vector should be roughly equal to the local concentration of the other end of the same molecule. However, diluting the large fragments results in a low concentration of circularized DNA fragments, and yields of transformants by electroporation are directly proportional to input DNA concentrations (Dower et al. 1988; Taketo 1988). The greatest number of BACs per microliter ligation was obtained when approximately 60 ng large lettuce DNA fragments were diluted three times prior to ligation with 20 ng of dephosphorylated pEC-SBAC4. The decrease in the concentrations of the low-melting-point agarose and/or the Gelase buffer also may have contributed to better conditions for ligation and/or electroporation. Further dilution decreased the numbers of clones obtained.

Utility of BAC vectors with an unique *Eco*RI cloning site

Existing BAC vectors were modified to create an unique *Eco*RI cloning site for several reasons. The availability of another unique site provided the opportunity to reduce potential cloning bias that might result from using only *Hin*dIII. Also, partial genomic digestions using competition between *Eco*RI endonuclease and *Eco*RI methylase results in a narrower range of fragment sizes and should be more repeatable (Larin et al. 1991). In addition, the recent availability of *Tsp509*I, an endonuclease with a four-base recognition sequence that leaves cohesive ends with an *Eco*RI site (NEB), provides the possibility for generating partial digestions with minimal site bias.

BAC vectors with a unique *Eco*RI cloning site in combination with *Rec*A-assisted restriction endonuclease (RARE) cleavage (Ferrin and Camerini-Otero 1994) may allow the cloning of specific genomic fragments rather than the generation and screening of complete genomic libraries. RARE cleavage allows the cleavage of genomic DNA at specific sequences containing *Eco*RI sites. Although a variety of enzymes can theoretically be used, the current quality of commercially available methylases restricts RARE to protection using *Eco*RI methylase and digestion of specific sites using *Eco*RI endonuclease. To date, it has not been possible to clone large genomic fragments generated by RARE cleavage as the yields are small and yeast transformation efficiencies are too low for the generation of YAC clones. However, given the much higher transformation efficiencies into *E. coli*, it may be possible to clone the products of RARE cleavage as BAC clones. This would be particularly useful for completing contigs of genomic clones based on *Eco*RI fragments in BAC or YAC vectors as the terminal sequences will contain an *Eco*RI site.

AFLP™ fingerprinting (Vos et al. 1995) is another technique that interfaces with BAC vectors containing a unique *Eco*RI site. AFLPs in combination with bulked segregant analysis (Michelmore et al. 1991) or deletion mutants (Lavelle and Michelmore unpublished) provide the ability to saturate a region of interest with markers at a density at greater than one marker every 50 kb. As this is considerably less than the average insert size of BAC clones, libraries with average insert sizes of 100 kb or more should be more than adequate for map-based cloning without multiple walking steps. Also, as AFLP analysis frequently uses *Eco*RI as one of the two enzymes used to generate fragments, all such markers will end in sequences containing an *Eco*RI site, thereby providing the possibility of using RARE cleavage to release fragments between AFLP markers.

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