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E. Y. Yoo · S. Kim · Y. H. Kim · C. J. Lee · B.-D. Kim

Construction of a deep coverage BAC library from *Capsicum annuum*, 'CM334'

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Abstract A bacterial artificial chromosome (BAC) library consisting of 235,000 clones with an average insert size of 130 kb was constructed from *Capsicum annuum*, 'CM334'. Based on a pepper haploid genome size of 2,702 Mbp/C, the BAC library is estimated to contain approximately 12 genome equivalents and represents at least 99% of the pepper genome. Screening of the library with mitochondrial DNA probes (*cox*II, *cox*III, *atp*6 and *atp*9) and chloroplast DNA probes (*atp*B, *rbc*L) indicated that contamination with cytoplasmic DNA was less than 0.5%. To estimate the possibility of isolating a specific clone, the library was screened with single or low-copy gene-specific probes and RFLP probes. Screening of high density BAC filters with RFLP markers linked to *L* (TMV resistance), *y* (fruit color), *C2* (fruit color) and *C* (pungency) loci under high stringency conditions revealed that at least three positive BAC clones were found per each probe. This fact indicates that the library is highly reliable and represents a resource for map-based cloning, physical mapping, and characterization of upstream and downstream regulations of the chili pepper genes.

Keywords *Capsicum annuum* · BAC library

Introduction

Chili peppers have been widely used for the fresh market and processed food additives such as hot sauce, because of their unique pungency and fruit color. As the importance of pepper breeding grows, molecular genetic study of the pepper also developed. There are molecular genetic linkage maps with interspecific (Livingstone et al. 1999; Kang et al. 2001) and intraspecific (Lefebvre et al. 1995) populations in the *Capsicum* species. QTL loci of fruit-related traits (Ben Chaim et al. 2001) and disease resistance loci for PVY, TMV (Caranta et al. 1999; Grube et al. 2000a) and *Phytophthora capsici* (Lefevre and Palloix 1996) were assigned to the genetic maps.

The bacterial artificial chromosome (BAC) library has been proved to be a very valuable tool for map-based cloning in many plants. There was no pepper BAC library available. The haploid genome size of pepper is known to be about 2,702 Mbp/C (Arumuganthan and Earle 1991), which is almost the same as the human genome and 18-times bigger than the Arabidopsis genome. One hundred thousand clones are required to have a 99% probability of a particular clone represented in a library having an average insert size of 150 kb for pepper. Our group constructed a pepper BAC library that had an average insert size of 80 kb but the genome coverage was only three times (Yoo et al. 2001). At that time, only three probes among ten single-copy genes showed positive signals after hybridization of the BAC filters. We concluded it was insufficient for map-based cloning and physical mapping for the chili pepper, and decided to develop a deep-coverage BAC library containing over ten times the genome equivalents.

In this paper we report that a BAC library for chili pepper, 'CM334' has been constructed with 12 equivalents of the haploid genome. The library was characterized for the insert size and cytoplasmic DNA contamination. We tested the possibilities for map-based cloning of disease-resistant genes and fruit quality genes by hybridization with single gene-specific probes.

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E. Y. Yoo · S. Kim · C. J. Lee · B.-D. Kim (✉)
School of Plant Science, College of Agriculture and Life Sciences, and Center for Plant Molecular Genetics and Breeding Research, Seoul National University, 103 Seodoon-dong, Suweon 441-744, Korea
e-mail: kimbd@snu.ac.kr
Tel.: 82-31-290-2563, Fax: 82-31-296-2768

Y. H. Kim
National Institute of Agricultural Biotechnology,
225 Seodoon-dong, Suweon, 441-707, Korea

Materials and methods

Plant materials

Seeds of chili pepper *Capsicum annuum* 'CM334' were kindly provided by Dr. A. Palloix at INRA, France. The seeds were germinated and grown for 8 weeks under greenhouse conditions before nuclear isolation.

BAC library construction

Pepper nuclei were isolated as previously described (Zhang et al. 1995). After intact nuclei were obtained from young leaves, they were capsulated with agarose and partially digested with the restriction enzyme *Hin*dIII. Partial digests of the megabase-sized DNA were achieved as follows; chopped plugs were distributed in 50-μl aliquots and incubated on ice for 30 min with 7 μl of 10× enzyme buffer, 7 μl of 40 mM spermidine, and 1 μl of BSA. After 30 min incubation with two units of *Hin*dIII restriction enzyme on ice, the digestion reaction was allowed to proceed at 37 °C for 30 min. The partially digested megabase-sized genomic DNA was separated by pulsed field gel electrophoresis (PFGE; CHEF DRIII, BIO-RAD, USA). Size selection was based on: 1% low-melting temperature agarose, a 1–50 s linear ramp, 6 V/cm, 12 °C, a 22-h run time and 0.5× TBE buffer. After separation by PFGE, DNA fragments were first selected for the sizes between 100 and 300 kb, which was cut out and separated by PFGE for the second time. The gel-pieces containing DNA fragment between 100–250 kb were cut out and the DNA was electro-eluted out of the agarose gel. The single-copy BAC vector, pIndigo536, was obtained from Dr. R. Wing at Clemson University, USA. Ligation was performed with a 20-ng vector and 200 ng of DNA in a 150-μl reaction volume for 16 h at 16 °C. Recombinant DNA in a 2-μl ligation reaction mixture was transformed into a 20-μl competent *Escherichia coli* cell (DH10B) at 1.3 kV, 25 μF and 200 ohms, using the BIO-RAD electroporation system. Transformed cells were then diluted immediately with 0.75 ml of SOC and incubated at 37 °C for 45 min before being plated on selective medium (LB, Luria-Bertani medium) containing 12.5 μg/μl of chloramphenicol, 0.55 mM of IPTG and 80 μg/ml of X-Gal. White colonies containing recombinant DNA were picked individually with toothpicks or an automatic picking system, Biopick (BioRobotics, Cambridge, UK), to 384-well microtiter plates (Incyte) containing 70 μl of freezing broth (Woo et al. 1994). After overnight incubation, the library was duplicated and stored in two different –80 °C freezers.

BAC library screening

For hybridization-based screening of the library, bacterial colonies were placed on a nitrocellulose membrane using the automatic gridding system, TAS (BioRobotics, Cambridge, UK). The clones were placed on the grid in double spots using a 4 × 4 array with six fields per 22 × 22 cm of nitrocellulose filter (NEN, USA). After inoculation, the membranes were incubated at 37 °C for 12–18 h to bring the size of the bacterial colonies to 2–3 mm in diameter. The bacterial colonies were then lysed by the method of Cold Spring Harbor Laboratory. The library of 235,000 clones was plated and blotted onto 26 membrane filters. Probes (25 ng) were labeled by random priming using the rediprime-II Random Prime labeling system (Amersham Pharmacia Biotech, UK) and purified with Quick Spin Columns (Roche, Germany). The filters were put into five hybridization bottles with 30 ml of the hybridization buffer containing 1% BSA, 1 mM EDTA (pH 8.0), 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS and 10 μg/ml of salmon sperm DNA. After hybridization for 18–24 h at 65 °C, the filters were washed 3-times for 30 min each with increasing stringency of 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS; 0.5× SSC, 0.1% SDS; and 0.2 SSC, 0.1% SDS. The membrane filters were then autoradiographed using FUJI super RX film (FUJIFILM, USA) with a single intensifying screen at -80 °C for 24–48 h.

Results and discussion

Construction and characterization of pepper BAC library

The pepper BAC library was constructed through four main steps generally used: manipulation of high-molecular-weight DNA from pepper nuclei and preparation of BAC vector, ligation of the insert DNA and vector DNA, transformation of the ligated DNA into *E. coli* cells and characterization of the reliability for the constructed library. A total of 235,000 clones with a 98% insertion rate were obtained from one ligation mixture. Eighteen clones were picked randomly out of the library and were digested with *Not*I before separating by agarose-gel electrophoresis. As shown in Fig. 1(a) each clone produced bands for the vector and a single insert DNA without any contaminating band. The single insert DNA was somewhat expected because the pepper genome is rare for the *Not*I site, and in the pIndigo536 BAC vector *Not*I sites flank the *Hin*dIII insertion site. An autoradiogram of the gel after Southern-blot analysis with the total pepper genomic DNA as a probe revealed a clear single band confirming the purity and the pepper origin of the insert DNA [Fig. 1(b)].

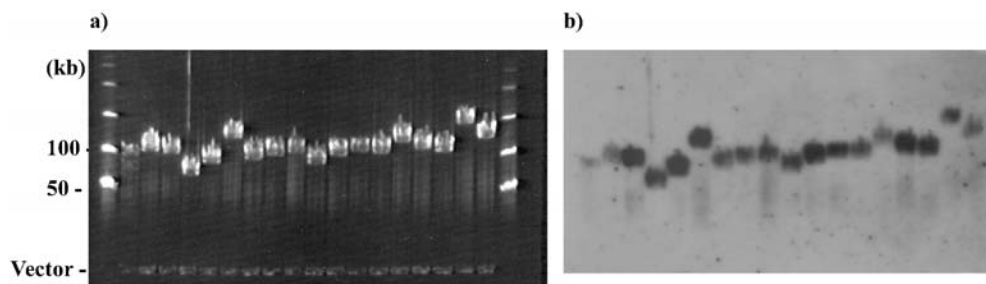

Fig. 1a, b Analysis of the BAC clones. (**a**) Ethidium bromide-stained agarose gel showing 18 random BAC clones digested with *Not*I and separated by PFGE (*lanes 2–19*). The sizes of DNA fragments were determined on the basis of lambda concatamer size-markers (*lane 1*) and the 7.3-kb pIndigo536 vector. The sizes of the BAC inserts ranged from 80 to 150 kb. (**b**) An autoradiogram showing Southern-blot analysis. The gel in (**a**) was transferred to a nylon membrane and hybridized with the [α-^{32}P] dCTP-labeled total pepper genomic DNA as a probe

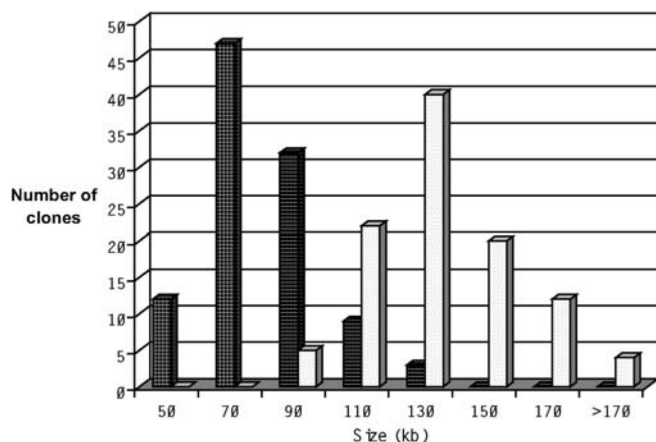

Fig. 2 Comparative graph of size distribution between the first- and the second-size selections. Each DNA isolated from each 103 randomly selected BAC clones was separated by PFGE for comparison between the first-time and the second-time size-selection steps. The *black bar* is for the first and the *white bar* for the second size-selection

In increasing the average insert size over 100 kb, the critical point was to cut out the gel and elute DNA fragments from 100 kb to 300 kb in size, not for one time, but for the second time (Woo et al. 1994). The partially digested pepper genomic DNA by *Hin*dIII was fractionated by PFGE, and the DNA fractions from 100 kb to 300 kb were selected for size twice to get rid of trapped small DNA fragments below 100 kb. One hundred and three BAC clones were chosen randomly and digested with *Not*I, and electro-separated by PFGE to estimate the average insert size. Figure 2 shows the improvement of the insert size after the second size selection. The insert sizes ranged between 50 to 130 kb with the average of 80 kb after the first size selection, whereas after the second size selection the insert sizes ranged between 80 to 300 kb with the average of 130 kb.

The average insert size, 130 kb, of the BAC library consisting of 235,000 clones constitutes about 12 genome equivalents based on the average insert size of a haploid genome of 2,702 Mb (Arumuganthan et al. 1991). This size of the BAC library suggests that the probability of recovering any specific sequence of interest is higher than 99%. To determine the degree of contamination of cytoplasmic DNA in the BAC library, mitochondrial DNAs of chili pepper such as *cox*II, *cox*III, *atp*6 and *atp*9 were used as probes to screen the library. The screening result revealed that the mitochondrial DNA contamination in the library was 0.03%. Chloroplast genes of barley such as *atp*B and *rbc*l were used as probes for screening, which showed 0.5% contamination. Thus, the contamination rate by total cytoplasmic DNA was less than 0.5%.

BAC clones containing gene-specific probes

The whole BAC library was picked up on 614 384-well microplates and then gridded onto 26 22 × 22 cm nylon-membranes. Completeness of the library was tested with two cDNAs, four RFLPs and one AFLP marker as single or low-copy probes (Table 1). At least three positive BAC clones were identified for each probe.

BAC clones for *CCS, PSY* and *LCYB* were identified. These structural genes involved in the carotenoid biosynthesis pathway had been identified in pepper (Huh et al. 2000) and located on the SNU pepper linkage-group 4, 7 and 10, respectively (Kang et al. 2000). *CCS* is tightly linked with the yellow/red locus (Lefebvre et al. 1998) and *PSY* is tightly linked with the orange/red locus (Huh et al. 2000). Tightly linked genetic markers, functionally characterized loci and large-sized insert DNAs could increase the probability of success for the map-based cloning. The Mexican chili 'CM334', the plant material for this BAC library, contains several interesting genes such as *C* conferring pungency, and disease resistance genes against *P. capsici*, TMV and PVY. It was noteworthy that one tomato RFLP marker, TG205, which supposedly has a lower homology to the pepper genomic sequence also produced three positive BAC clones. Using markers such as TG205 (Blum et al. 2002) and SB2-66 (Kim et al. 2001), which are known to be linked to the *C* locus, three of each BAC clones were identified. Further characterization of these clones is in progress. Two markers, CDI136 and E35/M48-101, which are linked to the TMV-resistance locus *L* on pepper linkage group 11, were used as probes for screening the BAC library to produce 6 and 226 positive clones, respectively (Table 1). The *L* locus, where many complex disease resistance genes are linked to, is known to be located in the telomeric region of chromosome 11 and the region exhibits many repetitive DNA sequences (Grube et al. 2000b). Hence, it would be very hard to map this region for saturation with molecular markers because of the low

Table 1 Identification of BAC clones corresponding to gene-specific and RFLP probes of chili pepper

Probe	Plant, marker	Copy number	Hits of BACs	Source and reference
CCS	Pepper, RFLP	1	3	Capsantin-capsorubin synthase (Huh et al. 2001)
PSY	Pepper, cDNA	1	8	Phytoene synthase (Huh et al. 2001)
LCYB	Pepper, RFLP	1	4	Lycopene β-cyclase (Huh et al. 2001)
TG205	Tomato, RFLP	1	3	Linked to *C* locus (Blum et al. 2002)
SB2-66	Pepper, cDNA	1	3	Putative capsaicinoid synthetase (Kim et al. 2001)
CDI136	Pepper, RFLP	3	6	Linked to *L* locus (not published)
E35/M48-101	Pepper, AFLP	5	226	Linked to *L* locus (Ben Chaim et al. 2001)

polymorphism displayed. The BAC clones might be used to fine-map, and the markers made from the BAC-ends could be used for the initial step of positional cloning.

Applications of the BAC library

Large insert DNA libraries have proven very useful for many plant genomic applications. The YAC library of bell pepper representing three haploid genome equivalents was used in map-based cloning of the *Xanthomonas*-resistance gene *Bs2* (Tai et al. 1999). The first batch of the BAC library covered three equivalents of the chili pepper haploid genome (Yoo et al. 2001) and the average insert size was relatively small, about 80 kb. This average insert size may not be big enough for map-based cloning on the basis of the relationship between the genetic and physical distance of pepper, which is approximately 250 kb to 2.5 Mb per 1 cM (Lefebvre et al. 1995; Tai et al. 1999). The deep coverage (12 ×) BAC library was constructed in this study. This pepper BAC library could be a very useful resource for map-based cloning of important genes and for pepper genomics. The BAC library described in this paper will be made available for distribution to academic researchers through the Center for Plant Molecular Genetics and Breeding Research (PMGBR) at Seoul National University (http://www.plaza1.snu.ac.kr/~cpmgbr).

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