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Progress in Natural Science 18 (2008) 143–147
Progress in Natural Science

Construction of a BAC library from cucumber (*Cucumis sativus* L.) and identification of linkage group specific clones

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Received 18 June 2007; received in revised form 6 September 2007; accepted 7 October 2007

Abstract

A bacterial artificial chromosome (BAC) library consisting of 19,200 clones with an average insert size of 105 kb has been constructed from a cucumber (*Cucumis sativus* L.) inbred line S94, derived from a cultivar in North China. The entire library was equivalent to approximately 5 haploid cucumber genomes. To facilitate chromosome engineering and anchor the cucumber genetic linkage map to its chromosomes, 15 sequence-characterized amplified regions (SCAR) and seven simple sequence repeats (SSR) markers from each linkage group of cucumber were used to screen an ordered array of pooled BAC DNA with polymerase chain reaction (PCR). Fifteen markers gave at least two positive clones. As a result, 22 BAC clones representing 7 linkage groups of cucumber were identified, which further validated the genome coverage and utility of the library. This BAC library and linkage group specific clones provide essential resources for future research of the cucumber genome.

Keywords: Cucumber; BAC library; Molecular markers; PCR screening; Specific clones

1. Introduction

Cucumber (*Cucumis sativus* L., $2n = 2x = 14$) is one of the important vegetable crops in the world. Because of its diversity of unisexual or bisexual flowering sex phenotypes [1], it is also an experimental model plant for sex determination studies. Cucumber has seven pairs of chromosomes and a haploid genome of 367 Mb, which is smaller than other species in Cucurbitaceae family, including melon (*C. melo* L.) and watermelon [*Citrullus lanatus* (Thunbrug) Matsumura and Nakai] [2]. Genome research of cucumber has made progress recently, and some cucumber genetic linkage maps have been constructed based mainly on randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), sequence-related amplified polymorphism (SRAP), sequence-characterized amplified regions (SCAR), some important agronomic traits and quantitative trait loci (QTL) [3–9]. These genetic maps are an invaluable resource that can be used not only to map important agronomic traits or QTL but also to anchor physical maps.

The bacterial artificial chromosome (BAC) library emerged as a preferred cloning system for physical mapping and genome analysis due to its low rate of chimera formation, stable maintenance of large DNA fragments, and easy manipulation of cloned DNA. BAC library is not only to facilitate map-based cloning [10,11], but also to provide unique opportunities for physical mapping, whole genome sequencing, and comparative genomics

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1002-0071/$ - see front matter
doi:10.1016/j.pnsc.2007.10.001

research. The first BAC library of cucumber reported was constructed with an inbred line derived in Japan with gynoecious sex expression and determinate growth habit characteristics [12]. However, the main cultivars planted widely in China have monoecious sex expression and indeterminate growth habit.

Although several cucumber linkage maps have been constructed, they did not contain numerous anchor markers. Fazio et al. constructed a cucumber genetic linkage map with most anchor markers including 24 SCARs and 14 SSRs in 7 linkage groups, and the number of anchor markers per linkage group ranged from 0 to 10, with an average of 5.4 [8]. We constructed a genetic linkage map of cucumber by F_2 population including 9 SCARs and 11 SSRs in 7 linkage groups, and the number of anchor markers ranged from 1 to 7 [13]. At present, the numbers of anchor markers have been added to 2–10 with an average of 5 per linkage group in our genetic map (data unpublished). The linkage groups on the genetic map of cucumber still cannot be linked with their chromosomes. Therefore, these efforts provide opportunities for identifying linkage group specific clones. Cytological and molecular cytogenetic studies of cucumber have been carried out for several decades and progress made in establishment of a high-resolution karyotype using molecular cytogenetic DNA markers, C-banding analysis, and fluorescence *in situ* hybridization (FISH) [14–19]. In this paper, we report the construction of a BAC library from an inbred line of northern Chinese cucumber cultivars planted widely in China. Furthermore, we identified a set of cucumber linkage group specific BAC clones by anchor markers (SCAR and SSR) from the genetic linkage map. This will become the necessary cornerstone for connecting linkage group to its chromosome through hybridizing cucumber chromosome by BAC clones.

2. Materials and methods

2.1. Plant material

Seeds of cucumber inbred line S94 were obtained from School of Agriculture and Biology, Shanghai Jiao Tong University, China. This cultivar belongs to Northern China type which was planted broadly in China with resistance to downy mildew. The S94 plants have monoecious sex expression (*f* locus), indeterminate growth habit, large fruit wart and many spines.

2.2. Methods

2.2.1. Construction of BAC library

The S94 plants were germinated in a growth chamber. Young leaves of plants 4–7 weeks old were collected after the plants were treated in the dark for 48 h. The nuclei were isolated from 50 g leaf material and embedded in low melting agarose plugs [20]. The agarose plugs containing the nuclei were incubated in the lysis buffer for 48 h. The plugs were washed twice with Tris–EDTA, and subsequently stored in 0.5 M EDTA at 4 °C. The procedure of the cucumber BAC library construction was according to the protocol described in Refs. [21,22]. The BAC vector pIndigoBAC-5 *Hin*dIII (Epicentre, Madison, USA) was used for cloning. Vector and size selected DNA fragments were mixed at the molar ratio of approximately 7 (vector) to 1 (insert) in a volume of 50 μl. Ligation was performed at 4 °C overnight. The ligation solutions were then desalted and transformed into *Escherichia coli* DH10B (Invitrogen, Carlsbad, USA) competent cells by electroporation [21]. Transformed cells were plated on LB agar medium with 12.5 μg/mL chloramphenicol and incubated at 37 °C for about 16 h. To pool the BAC library, library was plated out on agar plates with a density of about 200 clones/plate. All colonies from one agar plate were transferred to one well of a 96-well microtiter plate with LB medium. In this way, one well contained a mixture of about 200 recombinant clones. Finally, 19,200 recombinant clones were stored in one 96-well microtiter plate.

To estimate insert sizes, 100 BAC clones were selected randomly and inoculated on LB medium containing 12.5 μg/mL of chloramphenicol. BAC DNA was isolated using the alkaline mini-preparation method, digested with *Not*I and separated by pulsed field gel electrophoresis (PFGE) [21]. Insert sizes were estimated according to the MidRange I PFG marker (New England Biolabs, Beverly, USA).

2.2.2. Pooling of the library for PCR screening

To facilitate PCR screening, we established a three-dimensional PCR screening system [22]. Two hundred clones in each well of a 96-well plate as 1-dimensional pools, and then clones belonging to the same columns or rows were pooled together. Twelve column pools and 8 row pools were produced. This screening strategy consists of two steps. In the first step, the DNA isolated from each pool by the alkaline lysis method was used as template for the first PCR screening. By means of first PCR screening, the location of positive clones was determined in a positive well on the 96-well plate. In the second step, the culture from this positive well was plated on LB agar medium with 12.5 μg/mL chloramphenicol and incubated at 37 °C for about 16 h to obtain single colonies. DNAs extracted from randomly selected colonies were used as templates for PCR screening to identify positive individual clones. PCR amplification was carried out with 0.5 μl of BAC DNA template in the presence of 200 μM of each dNTP, 2 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase, 1 × *Taq* polymerase buffer and 0.5 μM of each primer, in a final volume of 10 μl. Following initial denaturation at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 1 min was performed. PCR products were separated by 2.0% agarose gel electrophoresis.

Table 1
Characteristics of linkage group anchor markers used to screen the cucumber BAC library

Marker	Type	Size (bp)	Linkage group[a]	Source of reference
S_S13-2	SCAR	700	1	[13]
C162	SSR	269	1	[23]
AI4SCAR	SCAR	1200	1	Staub Lab[b]
AO12SCAR	SCAR	1300	1	Staub Lab[b]
CSWCT25	SSR	357	2	[25]
CS30	EST-SSR	151	2	[24]
BC403SCAR	SCAR	774	2	Staub Lab[b]
S_AV14	SCAR	494	2	[13]
S_BE5-3	SCAR	281	3	[13]
CS8	EST-SSR	180	3	[24]
S_AB14	SCAR	534	4	[13]
S_AU18-1	SCAR	409	4	[13]
CSWACC02	SSR	227	4	[25]
L19-2-SCAR	SCAR	1000	5	[8]
CS5	EST-SSR	299	5	[24]
AN5SCAR	SCAR	875	5	Staub Lab[b]
BC526-SCAR	SCAR	970	6	[8]
C1SCAR	SCAR	372	6	Staub Lab[b]
S_BC526-2	SCAR	219	6	[13]
BC515SCAR	SCAR	650	7	[8]
CSWCT11	SSR	226	7	[25]
S_H12	SCAR	767	7	In this study[b]

[a] Linkage groups as designated by Yuan et al. [13].
[b] Markers from unpublished reference.

2.2.3. Isolation of linkage group specific BAC clones

Twenty-two markers from the cucumber linkage map, which was constructed with F_2 population (data unpublished), including 15 SCAR and 7 SSR, were employed in this study. Five SCAR markers (AI4SCAR, AO12SCAR, BC403SCAR, AN5SCAR and C1SCAR) were provided by Dr. Staub (http://www.vcru.wisc.edu/staublab). Seven SCAR markers were derived from their corresponding RAPD markers, respectively, and six of them are described in Ref. [13]. S_H12 primer sequences are 5′-ACG CGC ATG TTT AGC TAC TT-3′ (S_H12F) and 5′-ACG CGC ATG TTA ACA CGA GA-3′ (S_H12R). Others including three SCAR markers and seven SSR markers are presented in Refs. [8,23–25]. All of these markers were used to amplify the corresponding fragments from genomic DNA of the inbred line S94 (Table 1).

3. Results

3.1. Cucumber BAC library construction and characterization

We constructed a BAC library for cucumber using genomic DNA of the inbred line S94. The library was constructed using the *Hin*dIII restriction enzyme and contains 19,200 clones stored into one 96-well plate. To estimate insert size of the cucumber library, 100 randomly selected BAC clones were analyzed by *Not*I digestion and PFGE. The result indicated that 90% of the clones contained an insert with an average size of 105 kb and insert size ranged from 80 to 140 kb (Fig. 1). Based on an estimated cucumber genome size 367 Mb, the whole DNA inserted in the library could be equivalent to approximately 5 haploid cucumber genomes.

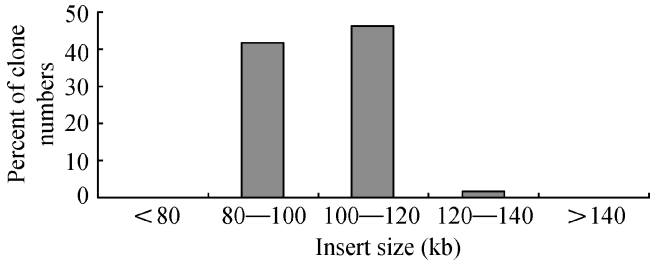

Fig. 1. Insert size distribution of 100 clones selected randomly from the BAC library of cucumber.

3.2. PCR screening of BAC library with linkage group anchor markers

To further evaluate quality of the library and isolate BAC clones specific for each linkage group of cucumber, 22 anchor markers from each linkage group were used to screen an ordered array of pooled BAC DNA with PCR. Using BAC DNA pool system, two rounds of PCR analysis were required to identify individual BAC clones. Fig. 2 shows a representative case of BAC screening using S_AV14 marker.

3.3. Isolation of BAC clones specific for linkage group

In the BAC library, at least one positive BAC clone could be detected with all tested markers. A total of 60 BAC clones were identified and the number of BAC clones detected by each marker ranged from 1 to 9 (Table 2). Fifteen markers gave at least two positive clones, respectively. Seven markers gave single positive clones individually. In order to use BAC clones as probe to hybridize cucumber chromosomes using FISH technique, 22 BAC clones representing 7 linkage groups of cucumber were selected from 60 BAC clones.

4. Discussion

We have constructed a cucumber BAC library consisting of 19,200 clones with an average insert size of 105 kb, thus representing approximately 5 cucumber haploid genome equivalents. This library provides a greater than 99% probability of obtaining a particular DNA sequence. The insert size of around 100–200 kb makes it an ideal source for physical mapping, FISH analysis to localize the position on chromosome, isolation of closely linked polymorphic markers, and identification of regulatory elements in gene expression studies [26]. In comparison with the first reported cucumber BAC library constructed with an inbred line derived from Japan which has gynoecious sex expression and determinate growth habit, this BAC library was

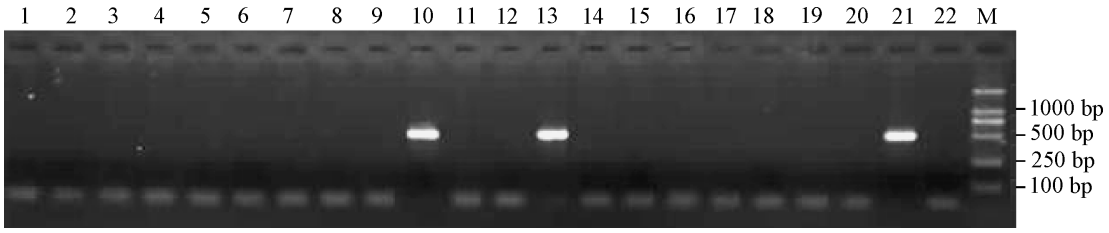

Fig. 2. PCR amplification of cucumber BAC DNA pools with the S_AV14 marker. Lanes 1–12, two-dimensional pools; lanes 13–20, three-dimensional pools. Cucumber genomic DNA in lane 21 represents the positive control, and 22 indicates the negative control (DNA replaced by ddH_2O).

Table 2
BAC clones of the cucumber library identified with linkage group anchor molecular markers

Marker	Number of BAC clones	Marker	Number of BAC clones
S_S13-2	2	S_AU18-2	2
C162	1	CSWACC02	3
AI4SCAR	2	L19-2-SCAR	4
AO12SCAR	2	CS5	1
CSWCT25	1	AN5SCAR	1
CS30	1	BC526-SCAR	5
BC403SCAR	2	C1SCAR	3
S_AV14	1	S_BC526-2	3
S_BE5-3	9	BC515SCAR	4
CS8	4	CSWCT11	5
S_AB14	1	S_H12	3
Total	60	Average	2.73

constructed from an inbred line S94 which has monoecious sex expression and indeterminate growth habit [12]. The S94 has been used as a parent to develop a recombination inbred line population. Therefore, the library constructed from S94 could be used directly to isolate genes and QTLs identified in the population, and anchor the cucumber genetic map to its chromosomes. The BAC vector has three restriction enzyme sites (*Eco*RI, *Bam*HI and *Hin*dIII) for cloning commonly [27]. Previously reported library was constructed by *Eco*RI and *Bam*HI, our new library being quiet different from that. Hence, construction of BAC library that has significantly larger insert sizes and wider genome coverage by using different restriction enzymes is essential for comprehensive genomics research of cucumber. Up to now the libraries constructed from different cucumber lines with different agronomic traits which rendered them complementary genomic resources for the scientific community working on this crop.

One of the most technically difficult parts of BAC library construction is the isolation of high quality high-molecular-weight (HMW) DNA. The nuclei were embedded in agarose plugs and then incubated in the lysis buffer [28]. Hence, sample collections will affect the quality of nuclei directly. We kept the plant in the dark for 48 h prior to sample collection to minimize the starch and polysaccharide content which would greatly affect the quality of HMW nuclear DNA isolation. To increase the average insert size, two rounds of size selection of partially digested HMW DNA were carried out. After the first electrophoresis, a part of the gel containing DNA fragments of 100–300 kb was excised from the gel, electroeluted and subjected to a second round of size selection. This second size selection was for further removal of smaller DNA fragments.

To increase efficiency of PCR screening, we designed a pooling strategy suitable for screening of this library using a wide range of PCR-based markers, which reduced the time and cost of experiment required in identification of candidate clones. A BAC library with 19,200 clones was stored in one 96-well plate, and one well contained a pool of about 200 clones. Screening BAC by RFLP or radioactive markers was inconvenient due to original clones in the library getting mixed. However, this pooling strategy provided facility to store and replicate the library, which simplifies the pooling procedure. Our row DNA pools contained about 2400 clones and the column DNA pools contained about 1600 clones in the library. After two rounds of screening with 22 markers, 60 positive clones were identified from the BAC library. This provides a possibility for locating linkage group to cucumber chromosome.

The problems for current cucumber genetic map are less anchor markers (5 markers per linkage group on average) in the map and uneven distribution of these markers on linkage groups [8]. In addition, the cucumber genome is composed of a significant amount of repetitive DNA sequences [12]. Therefore, these problems caused difficulties for identification of linkage group specific clones. In the PCR screening process, some markers gave more positives than expected. Whereas one or two markers gave no positive clones ascribed to have bias in genome coverage using a single enzyme. Consequently, only 22 markers from cucumber linkage map were used in this study. The new SCAR and SSR markers could be developed using the end sequence of the linkage group specific clones. It will produce new markers for gene mapping and integration of different cucumber genetic maps.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 30671111), Shanghai Municipal Scientific & Technological Commission (Grant No. 043919317) and Shanghai Leading Academic Discipline Project (B209). The authors thank Dr. Staub J.E. (University of Wisconsin-Madison) for his assistance with

provision of sequences of partial SCAR markers and helpful advice.

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