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RESEARCH ARTICLE

PCR-based screening of BAC clones of different chromosomes in Chinese cabbage

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Abstract **In this paper, taking SSR and functional gene sequence as the primers and the plasmid of first- and second-level pools of bacterial artificial chromosome (BAC) library as templates, the PCR method was used for specific clones of different chromosomes in Chinese cabbage. The results showed that the number of positive clones was 1–11 per primer and the average number of clone was 3.9 by screening 19200 clones of BAC library using 12 pairs of SSR primers from 10 linkage groups individually, which were nearly consistent with about 3.4 times of genome coverage. Positive clones were acquired in chromosome Nos. 2 to 5 and 8 to 10 without screening with the positive clones in chromosome Nos. 1, 6, and 7. In addition, the primer of *FLC1* functional gene of chromosome No. 10 was used for PCR screening, and two BAC clones containing *FLC1* gene were acquired. Therefore, different specific BAC clones of chromosomes were taken by using SSR primer and functional gene primer. Specific clone screening of chromosomes could provide a probe for identifying the chromosome accurately. Meanwhile, the BAC library screening method was optimized, serving as an effective technical means for quick BAC clone screening.**

Keywords Chinese cabbage, chromosome, SSR primer, mixing pool, PCR screening

Introduction

The chromosome is the carrier of biological genetic information, and different chromosomes have different functional genes. Therefore, there is significant meaning in breeding and studying the structure of chromosome, genetic information of each chromosome, and variation of biological character. DNA library is the basis of study on the structure of genome and its function. It is an important part in the study of eucaryon genome by cloning DNA molecules. In recent years, the large fractions of genome library are coming out in succession with the development of molecular biology. Bacterial artificial chromosome (BAC) library has been widely used due to its advantages such as large inserting size, strong stability, and easy to operate (Shizuya et al., 1992; Osoegawa et al., 1998). BAC library plays an important role in positional gene cloning (Giraudat et al., 1992), sequence analysis of genomes (Febrer et al., 2007; Terol et al., 2008), physical mapping (Mun et al., 2008; Szinay et al., 2008), gene location (Xiong et al., 2004; Huan et al., 2009), and comparative genomics (Achenbach et al., 2010).

The chromosome of Chinese cabbage belongs to A type, with the basic number of chromosome of 10. Its chromosomes are smaller than those of other plants. It is costly and time-consuming using the method of conventional karyotype analysis to identify the structure, and variation in number of chromosomes, or tracking alien chromosome and segments. This method also has the problem of low identifying precision on chromosome. The appearance of BAC-FISH technology has provided a shortcut for identifying the chromosome and its segments accurately. Therefore, it is vital of screening in specific BAC cloning of chromosome.

In this study, BAC mixing pool was built and PCR method was used to screen the specific BAC cloning of chromosome with the help of SSR markers from different linkage groups and specific functional genes as primer, providing a reliable marking probe for accurate identification of chromosome. In addition, the end sequencing of BAC clones from linkage group was carried out to develop the new fixed markers and

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provide as a solid foundation for future mapping integration and genetic positioning. Meanwhile, the BAC library screening method was optimized in this study, serving as an effective means for quick screening of positive clones containing functional genes.

Materials and methods

Experimental materials

The test material is the self-bred line '85–1' BAC library of Chinese cabbage provided by the vegetable breeding group of Horticultural College of Agricultural University of Hebei. The library was frozen in the form of monoclonal antibody in one hundred fifty 384-well plates (Nos. 1–150). Fifty 384-well plates (about 3.4 times of genome coverage) were selected as the materials for pool building and clone screening in this study.

Primer design and PCR screening conditions

Two types of primers were used in this experiment: one was selected from the SSR primer with linkage group of Chinese cabbage (Li, 2010) (Table 1). The other was based on the sequence of specific functional gene of chromosome. The functional gene primer was designed by the flowering-related gene *FLC1* sequence (Schranz et al., 2002) of A10 (chromosome 10) linkage group of Chinese cabbage in Genebank. All primers were synthesized by Shanghai Sangon Biology Engineering Co., PCR-based reaction system (20 μL): 100 ng DNA template, 1 U *Taq* DNA polymerase, 1 μL dNTPs (2.5 mmol/L), and 1 μL positive and negative primers, respectively.

Condition of amplification reaction was an initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 45 s, 55–59°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Building of BAC library pools

The method described by Liu et al. (2006) was used as reference for BAC library pools building, and some improvements were done.

First-level pool building

The prepared LB solid medium was put into the culture vessel, and the aseptic 384-reproducer picking up bacterial colony from the 384-well plate containing BAC clones was cultured on the LB solid medium. The bacterial colony was cultured at a temperature of 37°C for 15 to 202 h. The bacterial colony was put into the 10 mL centrifuge tube, making one first-level pool. Then, other 384-well plates were processed in the same way, generating many first-level pools. In this experiment, 50 first-level pools were built, containing 19200 clones. The alkaline lysis was used to make the plasmid DNA for each pool.

Second-level pool building

The aseptic toothpick was used to pick up 24 clones from A to P lines of 384-well plates containing positive clones. The clones were put into the 5 mL centrifuge tube containing 2 mL LB liquid medium and cultured at a temperature of 37°C and 225 r/min for 15 to 20 h. The bacteria liquid of each row was taken as the second-level pool, and each 384-well plate corresponding to first-level pool constructed 16 second-level pools. Each second-level pool contained 24 BAC monoclones. The plasmid was extracted from the second-level pool for use.

Monoclonal culture

A toothpick was used to pick up 1 to 24 monoclones containing positive clones, and the clones were cultured overnight to extract the plasma.

Positive clone screening and electrophoresis detection

The preparative pools and monoclonal plasma were taken as the template for PCR amplification. The PCR products were analyzed by 1.5% agarose gel electrophoresis (AGE) under a steady voltage of 120 V.

Table 1 SSR primer

No. of chromosome	No. of corresponding linkage group	SSR primer	Repeat motif
1	A09	Na10A08	$(CT)_{21}$
2	A03	Na10F06 BRMS-042	$(CCG)_6$ $(AAT)_4$,$(CT)_4(T)_2(CT)_4$
3	A01	ENA28	—
4	A06	ENA19	—
5	A05	ENA17	—
6	A02	Ol13E08	$(CT)_{11}$
7	A07	Ra2A05b	$(GT)_{47}$
8	A04	ENA3	—
9	A08	BRMS-033	$(CA)_{11}$
10	A10	ENA18 Ni3G04b	— $(AG)_{18}$

Results and analysis

BAC clones of different chromosomes screened by SSR primer

In this experiment, the first- and second-level pools were built to screen 19200 clones in fifty 384-well plates. Positive clones were screened by three steps of PCR amplification. If multi-positive first-level pools are taken by screening and the building of second-level pool can be increased, finally multi-positive monoclones can be screened.

According to the above screening procedure, 12 pairs primers were used for PCR-based screening on 19200 clones of Chinese cabbage. Figure 1 shows the screening results of No. 2 chromosome. Na10F06 primer can amplify the target lanes in clone 14 (Fig. 1C) of lane D (Fig. 1B) in No. 92 pool (Fig. 1A). The process has gone through three steps of PCR, 90 times of PCRs, to acquire one positive clone named 92D14. Other primers were screened by the same method.

PCR screening results of 12 pairs of SSR primers are as follows. The positive clones were taken in chromosome Nos. 2 to 5 and 8 to 10, while there were no positive clones in the screening of chromosome Nos. 1, 6, and 7. The number of positive clones was 1 to 10 per primer, and the average number of clone was 3.9 by screening 19200 clones of BAC library using 12 pairs of SSR primers from 10 linkage groups individually, which was nearly consistent with about 3.4 times of genome coverage. The primer ENA28 could screen 11 clones, while no clones were found in primers Na10A08, Ol13E08, Ra2A05b, and Ni3G04b (Table 2). Therefore, it is seen that some types of primers cannot screen any clones when using SSR primer to do screening of specific clones of chromosomes. As a result, more pairs of primers should be selected, or integration with other marking primers should be considered to use.

Table 2 BAC clones screening by different linkages of SSR primers

No. of chromosome	Marker name	Screening clone number
1	Na10A08	0
2	Na10F06	1
	BRMS-042	4
3	ENA28	11
4	ENA19	3
5	ENA17	1
6	Ol13E08	0
7	Ra2A05b	0
8	ENA3	4
9	BRMS-033	6
10	ENA18	1
	Ni3G04b	0

The average number of clone = total of screening clones/the number of markers getting clones.

Screening of functional gene primer

FLC1 gene has been known to exist on chromosome No. 10 of Chinese cabbage. *FLC1* gene was used to design the primer for PCR-based screening. Then, BAC clones containing *FLC1* gene from chromosome No. 10 could be acquired. According to the above screening system, there were amplification products in Lanes I and L of No. 146 pool. PCR amplification was carried out on each monoclone of Lane L, and the No. 11 monoclone in Lane L was amplified to the target band (Fig. 2). The same method was taken on No. 20 monoclone in Lane I, amplified to the target band. Two FLC1 monoclones were acquired through the above three steps of PCR amplification.

Discussion

The PCR method, applied to screen monoclones from thousands of clones, is a huge and complicated job. Therefore, BAC library screening method should be simple and easy to operate. Guan et al. (2008) built 3D pool in the study of BAC library screening of cucumber. Two hundred clones in each plate of 96 orifice plates were taken as the one-dimensional pool, and then the orifice plate was processed on horizontal and vertical lines to construct the two- and three-dimensional pools. The PCR method was used to ensure the specific position of the target clones on the 96 orifice plates, and little bacteria liquid was picked up and applied on the

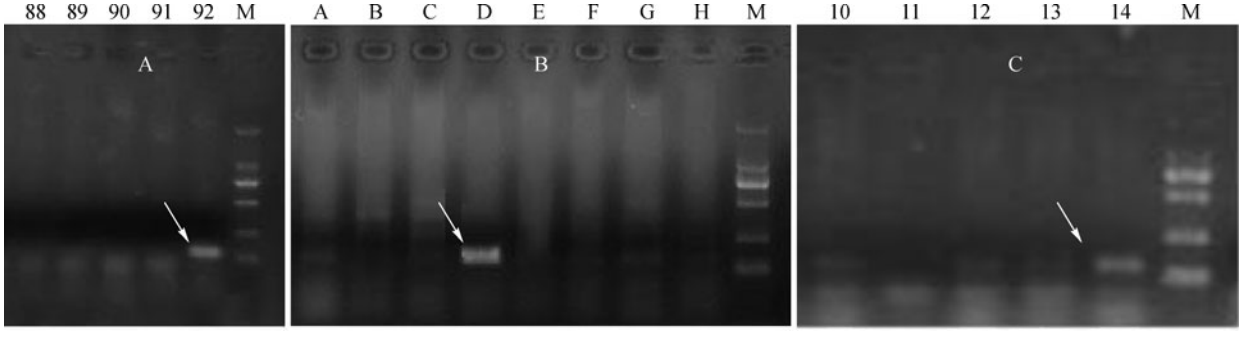

Figure 1 PCR-based screening of chromosome 2. M: Markers are 2000, 1000, 750, 500, 250, and 100 bp from top to bottom. A is first-level pool screening, B is second-level pool screening, and C is monoclonal screening.

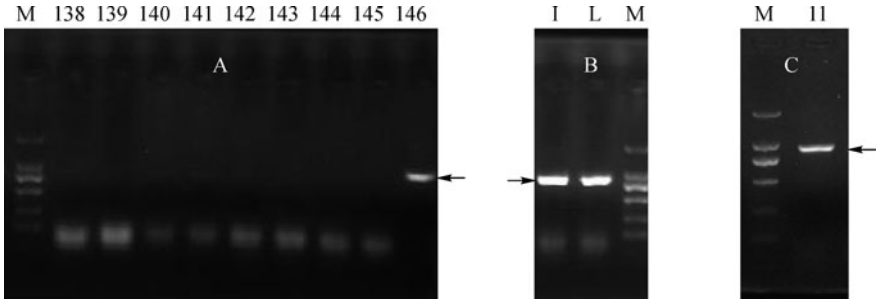

Figure 2 Positive BAC clone screening of chromosome 10. M are markers of 2000, 1000, 750, 500, 250, and 100 bp from top to bottom. A is first-level pool screening, B is second-level pool screening, and C is monoclonal screening.

plate. The clone was selected as the template to get monoclones using PCR method. The preservation of clones of this method was in the form of super pool. There was uncertainty on the number of clones when screening the monoclones. The multi-screened clones may be the same clone. In this paper, screening from 19200 monoclones to positive clones only needed 50 first-level pools, 16 second-level pools, and 90 times of PCR, acquiring one positive clone at least. This method was easy to operate, reducing the procedure of making mono-line pool and providing accurate screening results of monoclones.

When screening BAC library by PCR method, the selection of primer is vital. SSR is a kind of DNA molecular marker based on PCR technology, with features in abundant content, high polymorphism, preferred repeatability, and co-dominant inheritance (Luo et al., 2010). Therefore, SSR is an ideal tool for building genetic linkage map, genetic positioning, and screening of target trait molecular markers. The screening number of clones by PCR method is correlated with library cover, and the higher the cover is, the more the screening number will be. In this experiment, SSR primer was used to do BAC library screening, and the results demonstrated that some markers can screen over 10 clones, while some even cannot screen any clones, which accords with the study of Guan et al. (2008). There were three reasons for the result. First, there existed repetitive sequences in quantity of Chinese cabbage genome, and SSR was not the only copy in the genome, leading to the high cloning number. Secondly, restriction enzyme cutting site of library cloning led to no screening of clones in some markers. Thirdly, genome coverage was only about 3.4 times, leading to no screening of clones. Therefore, it is necessary for screening on multi-pairs of primers if the idiosyncratic BAC cloning of chromosome is wanted. Otherwise, the integration of SSR primer and other markers should be screened to acquire the target BAC clones.

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