PCR ANALYSIS WITH STS PRIMER: A TOOL FOR IDENTIFYING OVERLAPPING BACTERIAL ARTIFICIAL CHROMOSOME (BAC) CLONES IN RICE

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ABSTRACT

PCR analysis with STS primers was used to screen for overlapping clones and assembly of contigs with a BAC library from Azucena, IR64 nuclear DNAs comprising of 18,432 individual clones. One hundred twenty STS primers were surveyed with the pooled BAC DNAs and individual BAC DNAs. A total of 86 BAC clones were identified within 23 loci by 38 STS primers. Some contigs have been anchored on the rice chromosomes based on a STS marker map of IR64 / Azucena doubled haploid population. The results showed that PCR analysis with STS primers is a powerful tool in screening for overlapping BAC clones by generating many bands which are specific to IR64 genomic DNA and identifying BAC clones which carry these specific fragments.

Key words*:* PCR analysis, BAC, marker, cloning

INTRODUCTION

The development of BAC libraries has opened new avenues in physical mapping of plant genomes and in map-based cloning of agriculturally important genes. Yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), and phage artificial chromosome (PAC) are the major cloning systems capable of cloning large fragment of exogenous DNA in single clone (Woo et al. 1994, Schmitt et al. 1996). With these systems, physical maps of chromosome or chromosome regions have been constructed with overlapping clones. Several strategies have been used to generate overlapping clones. PCR can be used to screen YAC or BAC for overlapping clones. A physical map of chromosome 4 was constructed in *Arabidopsis* based on colony hybridization with the YAC library (Schmidt et al. 1995). STS analysis has also been used for the identification of YAC clones (Pillen et al. 1996a). Specific YAC clones can be identified by PCR through sequential analysis of DNA isolated from pooled BAC clones (Pillen et al. 1996b). In rice, both YAC and BAC libraries have been constructed (Umehara et al.1995; Wang et al. 1995; Yoshimura et al. 1996; Yang et al. 1997). RFLP and STS markers were used to identify the overlapping clones and assembly of contigs (Craig et al.1990, Green and Olson 1990). Genes were anchored on the restricted area, even the gene *Xa*-21 (bacterial blight resistance gene) was cloned from a BAC library (Wang et al. 1996). More than 60% of chromosome 6 was covered by YAC clones identified by RFLP markers (Ashikawa et al. 1996) while a YAC clone has been identified to carry *Xa*-1, another bacterial blight resistance gene (Yoshimura et al. 1996).

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PCR analysis with STS primer have been widely used in gene tagging and gene mapping such as: gene *tms*-3 (Lang.1997). PCR can be used to screen YAC or BAC for overlapping clones. Due to the limitation of the number of RFLP markers, physical mapping encounters problems such as the existence of big gaps and obstruction of repeat sequences. In an attempt to solve these problems, STS markers analysis was used in this study. We describe a strategy of using PCR with STS primers for screening overlapping BAC clones based on assaying DNA samples derived from pooled BAC clones. The high sensitivity, accuracy and productivity of this approach were demonstrated by successively scoring 86 positive clones within 23 loci by 38 STS primers.

MATERIALS AND METHODS

BAC library

A BAC library from IR64 nuclear DNAs was constructed at IRRI. A total of 18,432 clones corresponding to 3.28 rice genome equivalents were qualified and grown on 48 microtiter plates. Each plate is in an array of 384 wells with 16 rows and 24 columns. The insert size ranged from 37 to 364 kb with an average of 107 kb.

BAC DNA pools

Individual BAC clones were grown in 48 microtiter plates with 16 rows and 24 columns on each plate. Two levels of BAC DNA pools were prepared. The first level is on the entire library and the second level is on the individual plate. For the first level, three DNA pools were prepared, including 48 plate pools (384 clones each), 16 row pools (1152 clones each, 24 x 48), and 24 column pools (768 clones each, 16 x 48). Before DNA isolation, the BAC clones were grown to saturation before pooling. Thus there are a total of 88 first level DNA pools. For the second level, the bacterial cells from each row of a microtiter plate (24 clones) or each column of one plate (16 clones) were separately mixed together for DNA isolation. For each plate, there are 40 second level DNA pools (16 row pool and 24 column pool). For the entire BAC library of 48 microtiter plates, a total of 1920 second level pools were prepared.

Isolation of BAC DNAs

The putative clones were picked from the library and inoculated to 2 ml LB containing 12.5% μg/ml chloramphenicol and incubated at 37°C overnight. Miniprep of BAC DNAs follows method of Yang et al. (1997).

PCR analysis

PCR was run on Perkin Elmer 9600 (Perkin Elmer Cetus). A reaction mix of 20 μl contained 50 mM KCl, 10 mM Tris-HCl (pH 8. 3), 2. 5 mM $MgCI_2$, 0. 01% gelatin, 0.1 mM each of dNTPs, 20 ng of each primer, 30 ng of isolated AC DNAs, and 1 unit *Taq* polymerase. Amplification started with 2 minutes at 94^{o}C, followed by 40 cycles of 1 min at 94^{o}C (DNA denaturation), 1 min at 55-60 (primer annealing), and 2 minutes at 72^{o}C (primer extension). The reaction was terminated after 2 minutes of final extention at 72^{o}C. PCR products were separated in 1.5% agarose gel containing 0.5 μg/ml EtBr. Separated PCR products were visualized under UV light and photographed to examine the banding patterns of different samples.

Southern hybridization of the overlapping clones

Individual BAC DNA was digested by *Hin*dIII (which cut on the site of exogenous fragment and vector DNA). The generated products were separated on 0.7% agarose gel and transferred to Hybond-N+ (Amersham) nylon membrane by each overlapping group. The clone which produced more digestion fragments was further selected for the probe, labeling with α^{32}P-dCTP. Hybridization was done following the protocol of clone hybridization (Yang et al. 1997). After autoradiography, pairwise comparison of the banding patterns of putative overlapping clones was done.

RESULTS AND DISCUSSION

Identifying overlapping BAC clones with STS

The principle of identifying overlapping BAC clones with STS analysis. The 88 total DNA pools were first surveyed for overlapping BAC clones by STS primer which is known to produce multiple discrete PCR bands (DNA segments) from total genomic DNA isolated from IR64. For those DNA pools with BAC clones containing one of the segments, an PCR band corresponding the band from IR64 will be produced. Since this same BAC clone is in plate (P), row (R) and column (C) pools, the corresponding P, R and C pools will produce an PCR. If there is a second BAC clone which is overlapping with the BAC clone and shared the PCR locus, another set of P, R, C pools will produce same STS bands. Same banding patterns can be expected for other STS bands which were generated from the same primer. Similarly, we can infer the overlapping BAC clones from the STS banding patterns.

Surveying BAC library

One hundred twenty STS primers were used to survey the BAC library (Table 1). In the first round survey of the total BAC DNA pools, bands were obtained from each primer. Comparing with the control parent of IR64, additional bands in the pooled samples were observed. Due to differences in amounts of BAC clones, the band intensities varied and the size of amplified fragments range from 100 bp to 3,000 bp where in the fragments between 500 bp to 2,000 bp can be repeated well.

In the second round of verification, candidate overlapping clones were have by each band. A total of 200 clones were identified by PCR analysis with 60 primers. In the third round of verification, candidate overlapping clones were then grouped by each specific band. A total of 86 clones were identified by PCR analysis with 38 primers (Table 1). Some primers such as RZ792 have polymorphic bands can be efficiently checked with IR64 and Azucena.

Reconfirmation and assembly of contigs

Individual BAC DNA was digested by *Hin*dIII (Fig1), and southern hybridization was performed by each overlapping group. A total of 86 overlapping clones were found to be positive based on the hybridization patterns which show at least two identical bands pairwisely (one of them is the signal for the vector) (Table 1)

For example in the analysis of the positive overlapping clones, three clones (05P16, 20A 13 and 21C01) were found repeatedly present in third second round with primer RZ792. The first round BAC clone contains 4 plate(P5, P20, P21, P23), 4 row (A, B, C, P) and 3 columns (C1, C13, C16) which have the same amplified fragment of 650 bp by primer RZ792. The second round contains 3 clones (05P16, 20A3, 21C01) which have the same amplified fragment (Fig.2). The third round contains 3 clones (05P16, 20A3, 21C01) which have the same amplified fragment (Fig.3). Results showed that PCR is feasible and efficient:

Three steps of pooling method make STS easy and reliable in screening BAC library with a high accuracy. More information accumulated from STS analysis make two or more contigs automatically linearly ordered. Amplified fragments by STS can be easily mapped on chromosomes based on the genetic analysis with one segregating population. They can be the anchors for the overlapping groups. Another advantage of STS is that new fragments were amplified in the first round of BAC DNA pools which are not present in the control parent IR64.

Table 1. BAC contigs identified by STS marker

No	Marker	BACs	Insert size	Other markers' presence
1	RG457	07I22	1200	
		16C14		
2	RG171	08EÏ6	700	
3	LP0039/0040	21M01	350	
4	RFLP55	18J13	371	
5	LP0075/0076	08 I 21	580	
		08 G21		
6	LP0111/0112	23D09	450	
		23D06		
		23H03		
		23 I 13		
		23D05		
		23J13		
		23H09		

Table 1. (continued)

No	Marker	BACs	Insert size	Other markers' presence
7	C1027	20F13	238	
		34F07		
8	C1269	42F11	155	
9	C0496	42F11	144	C1269
10	C0757	42 A 21		
		42A01		
11	LP0146	07M07	200	
12	C0252	43N10	144	
13	RG28L/L	29L08	900	
14	RG220L/L	27N16	900	
15	LP0445/0446	17C11	200	
16	C0802	48F09	200	
17	RFLP62	02O 24	580	
18	LP0055/LP0056	23G08	920	
19	LP0085/0086	36J22	310	
20	LP0101/0102	01G15,43B22	220	
		04B24,43G23		
		37A 20,43A 23		
		37B20,43B23		
		37G20,43G23		
		37A 21		
		37 B21		
		37B 23		
		37G 23		
		37A 24		
		37B 24		
		37G 24		
		39G 22		
		43A 15		
		43B 15		
		43B 21		
21	RG29	26 I 15	700	
		26L24		
		26L15		
22	RZ244	44H12	650	
23	RZ476	20F05, 24P05	1050	
		20M05,27F05		
		20P05,32F05		
		24M05		
24	RZ527	25G05,38G13	1200	
		25G13,45G12		
		37G13		
25	RZ792	05P16	650	
		20A 16		
		21A 16		

Table 1. (continued)

No	Marker	BACs	Insert size	Other markers' presence
26	RZ957	36L07	3000	
27	RZ945	04H01 14A 16	1000	
28	RZ142	07D13, 16K07	450	
29	RZ14	07C24	1000	
30	RG144	10C01	1000	
33	RZ192	16K 19, 16K20	300	
34	RG163	07J04	600	
35	RG449	08G02	1800	
36	RG533	02E 22	700	
37	RG125	18L23,41K05	1100	
38	LP0115-0116	05 I 12	200	

Although some of the contigs identified by STS analysis can be anchored on chromosomes based on the genetic map of the amplified fragments, the efforts by RFLP and STS analysis were always needed. The known locations of these arbitrary primers on the 12 chromosomes of rice (Huang et al. 1997) make them well used as the anchor site for the overlapping groups.

The results of this study have proven the applicability of STS to be an added approach in the construction of the rice physical map which will be the basis for map-based cloning of genes. These cannot be cloned using the present biotechnological approaches (such as mRNA or protein-based gene cloning).

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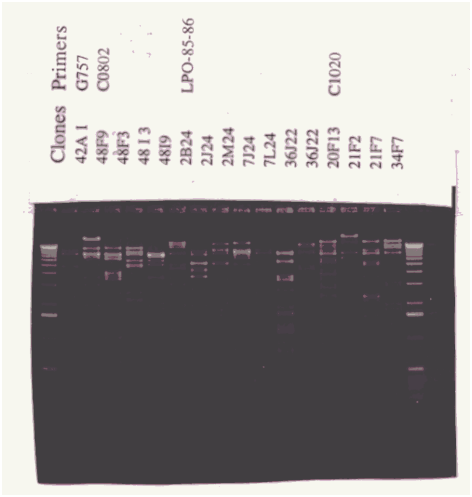

Fig 1. Hind III restriction enzyme analysis of the 16 BAC clones

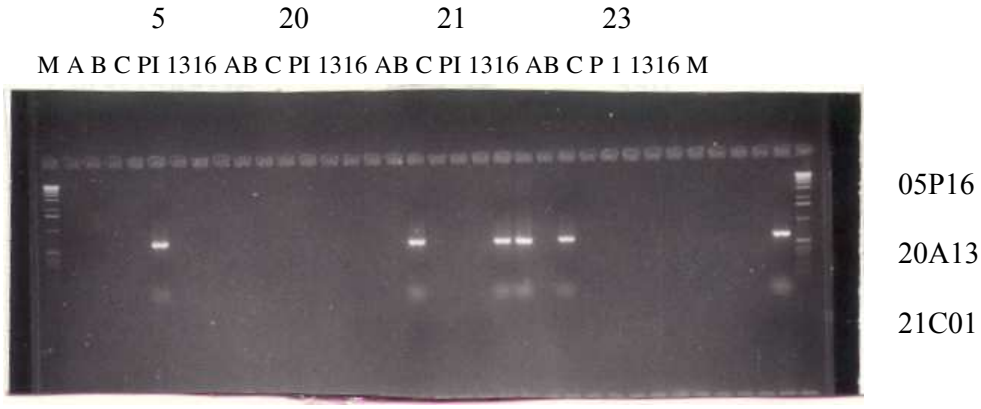

Fig 2. Second round screening (STS Sub BAC pool amplification) with primer RZ792

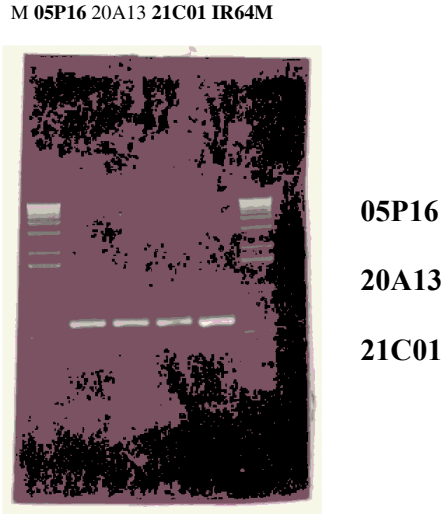

Fig. 3. Third round screening (STS individual BAC amplification) with primer RZ792

TÓM TẮT

Phân tích PCR với STS marker công cụ xác định các clones có tính chồng lấp trong vectơ "BAC" của cây lúa

Phân tích PCR với STS marker đã được sử dụng để thanh lọc các clones có tính chất chồng lấp, và hình thành những "contig" của kho kưu trữ "BAC" từ DNA của giống lúa Azucena và IR64. Vectơ BAC được hình thành như nhiễm thể nhân tạo do vi khuẩn (bacterial artificial chromosome). Contig là một đoạn dài sequence được hình thành từ một số các đoạn phân tử ngắn, chồng lấp nhau của sequence ấy. Kho lưu trữ này gồm có 18.432 clones.Chúng tôi thực hiện điều tra 120 STS primer với phân tử DNA của BAC lưu trữ và DNA của BAC thuộc từng cá thể. Tổng số dòng BAC clone đã được phân lập là 86 dòng, trong 23 loci của 38 STS primer. Một vài contig đã được "neo" giữ trên những nhiễm thể cây lúa trên cơ sổ bản đồ STS marker của quần thể đơn bội kép, từ cặp lai IR64 / Azucena. Kết qủa cho thấy: phân tích PCR với STS primer là một phương tiện rất tốt để tìm ra những BAC clone có tính chất chồng lấp, bằng cách tạo ra những băng rất chuyên biệt với DNA của IR64. Phương pháp này cũng xác định được các BAC clone mang những đoạn phân tử chuyên biệt như vậy.