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- Note -

Development of Simultaneous YAC Manipulation-Amplification (SYMA) system by Chromosome Splitting Technique Harboring Copy Number Amplification System

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Artificial chromosome manipulation and amplification of single-copy yeast artificial chromosome (YAC) are usually required in order to use YACs for applications such as physical mapping and functional analysis in eukaryotes. We designed and implemented a Simultaneous YAC Manipulation-Amplification (SYMA) system that combines the copy number amplification system of YAC with a convenient YAC manipulation system. To achieve the desired split and to amplify a YAC clone-harboring plant chromosome, a pBGTK plasmid containing a conditional centromere and thymidine kinase (*TK*) gene was constructed as a template to amplify the splitting fragment via PCR. By splitting, new 490-kb and 100-kb split YACs containing the elements for copy number amplification were simultaneously generated from a 590-kb YAC clone. The 100-kb split YAC was then successfully amplified 14.4-fold by adding 3 mg/ml sulfanilamide and 50 μg/ml methotrexate (S3/M50) as inducing substances.

Key words : Yeast artificial chromosome (YAC), PCR-mediated chromosome splitting (PCS), copy number amplification, *GAL1* promoter, thymidine kinase (*TK*) gene

Introduction

The yeast artificial chromosome (YAC) system has been successfully employed to map genetic markers and construct chromosome-specific YAC contigs of the *Trypanosoma cruzi* genome [12] and to assist in the assembly of the *T. cruzi* genome via shotgun sequencing [5,7]. Most YACs that have been studied contain single, contiguous, faithfully propagated DNA fragments [8,11], but unstable sequences are sometimes encountered [9]. Therefore, the development of a method to construct physical maps made up of overlapping YAC molecules is required [6,10,13]. However, there are limitations due to the single copy number of artificial chromosomes and the relative complexity of the eukaryotic genome compared to that of the prokaryotic genome. If an effective amplification system for YACs were available, the physical mapping and assembly of the genome would be simplified, which in turn may be decisive for determining the feasibility of YAC DNA transfer to eukaryotic cells for functional analysis.

Smith *et al.* reported the incorporation of elements allowing YAC copy number amplification, such as the conditional centromere and thymidine kinase (*TK*) genes, in yeast [14]. However, target-specific sequences necessary for splitting must be newly cloned into a YAC splitting vector harboring amplification elements, pCGS966, for each splitting event, which is prohibitively time-consuming. In a previous study, we developed an effective PCR-mediated chromosome splitting (PCS) method that can generate any targeted region of a plant chromosome without endogenous *ARS* elements as a new YAC [4]. The PCS method is a simple, efficient method that splits YAC-harboring plant chromosome regions as well as natural yeast chromosomes [4,15]. In this study, the YAC amplification system was combined with the PCS method to simultaneously introduce an amplification device during the YAC splitting process. We successfully used this system to amplify a newly generated YAC-harboring targeted plant chromosome region.

Saccharomyces cerevisiae SH6173 (*MATa ura3-52 his3-Δ200 leu2Δ1 lys2-1* (*or lys2Δ202*) *trp1* (*or trp1Δ63*) [*YAC CIC9e2*]) was used as a model strain for chromosome manipulation. The YAC clone CIC9e2 harbors a 590-kb region from *Arabidopsis thaliana* chromosome 5 [1] and was provided by Ohio State University (*Arabidopsis* Biological Resource Center, Columbus, OH, USA). The YPAD nutrient medium and synthetic complete (SC) medium for *S. cerevisiae* and cultivation methods were described in a previous report [3]. To amplify the split YAC, the amplification medium contains 0.67% (w/v) yeast nitrogen base, 1% (w/v) casamino

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acids, 3% (w/v) galactose, 50 μg/ml methionine, 0.04% adenine, 0.8 mg/ml thymidine, and various amounts of sulfanilamide and methotrexate [standard amounts are 1 mg/ml sulfanilamide and 10 μg/ml methotrexate (S1/M10)]. Yeast transformation was performed according to the high efficiency transformation protocol [2]. Approximately 5-10 μg of PCR fragments were used to split the plant chromosome cloned as a YAC.

To generate each splitting fragment, pSKCHY (*loxP-CgHIS3-loxP*) [4] and pBGTK were used as template plasmids for PCR. The pBGTK (Fig. 1A) plasmid, which includes components for amplifying split YACs, was constructed by inserting a 2.5-kb *Not*I fragment containing the Km^r gene as a selective marker for plant transformants from pKI01 [3] and inserting a PCR product containing the 2.1-kb *TK* gene and 1.4-kb *GAL1/CEN4*, respectively, into a pBluescript II SK+ vector. A PCR product containing the *TK* gene with a flanking *Bam*HI-*Not*I site was generated using pCGS966 [14] as a template and TK-F3 and TK-R3 as forward and reverse primers, respectively, and a PCR product containing *GAL1/CEN4* with a flanking *Kpn*I site was generated using pCGS966 [14] as a template and GC4-F and GC4-R as forward and reverse primers, respectively. The oligonucleotides used are listed in Table 1, and some of the primers were listed in Kim *et al.* [4]. Other methods, i.e., CHEF gel electrophoresis and Southern hybridization were performed as described in Kim *et al.* [3]. The intensity of hybridization signals with each probe was measured using the Scion image program.

Table 1. List of oligonucleotides used in this study

Oligonucleotides	Sequences (5′→3′)
TK-F3	GGATCCCGTGCCGCAGATCT
TK-R3	GCGGCCGCCTGGGTCGTCCACC
GC4-F	CCTAGGGGATTAGAAGCCGC
GC4-R	GGTACCGAATTCCCTTTAGT
Tr6-Am	$(CCCCAA)_6$ TAATACGACTCACTATAGGG

To split YAC CIC9e2 100 kb from the right end, two splitting fragments harboring each target sequence for homologous recombination were amplified as follows. One splitting fragment (2.2 kb), Am-I (Fig. 1B), consisted of the *CgHIS3* gene, a yeast transformant selective marker; a telomeric 5′-$(C_4A_2)_6$-3′ repeat sequence; and a target sequence that corresponded to a sequence from nucleotide position 448,711 to 449,100 of YAC CIC9e2; this fragment was prepared by the same procedure and using the same primers as used for amplification of the 100-I fragment described in a previous report [4]. The other splitting fragment (6.4 kb), Am-II (Fig. 1B), was amplified by two rounds of PCR as follows: a 6-kb PCR product containing *GAL1/CEN4* (GC4), *TK*, Km^r, and the telomeric 5′-$(C_4A_2)_6$-3′ repeat sequence was amplified by a first PCR using pBGTK as a template and SK-F and Tr6-Am as primers. Separately, a 400-bp target sequence corresponding to the sequence from nucleotide position 449,101 to 449,500 of the YAC CIC9e2 clone was amplified using genomic DNA from SH6173 as a template and 100 kb-3 and 100 kb-4 as primers. Then, a second PCR was performed using the 6-kb and the 400-bp PCR fragments as templates and Tr6-Am and 100 kb-4 as primers to generate the second 6.4-kb splitting fragment. By using two rounds of PCR, two splitting fragments, Am-I and Am-II, were successfully generated. To introduce an amplification system that could perform simultaneous chromosome splitting, the two splitting fragments were purified and transformed into

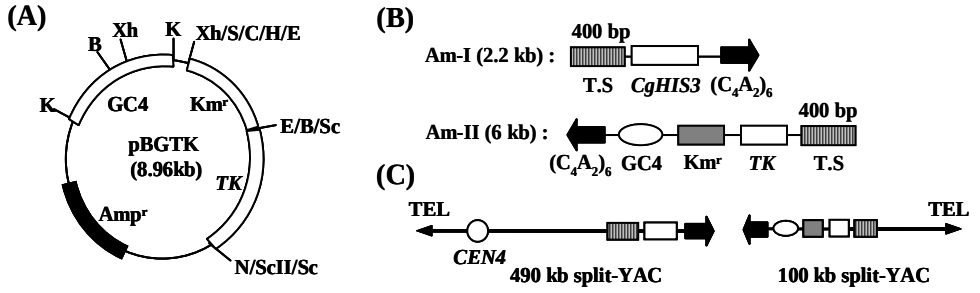

Fig. 1. Schematic diagram of pBGTK plasmid as a template for PCR (A), structures of splitting fragments (B) and split YAC generated from CIC9e2 YAC clone by splitting (C). Symbols: GC4, *GAL1/CEN4*, *GAL1* promoter/Centromere of yeast chromosome IV fusion; Km^r, the kanamycin resistance gene; *TK*, the Herpes Simplex Virus type 1 thymidine kinase gene; *CgHIS3*, *Candida grablata HIS3* cassette carrying the *loxP* sequences; T.S, target sequence; TEL, *Tetrahymena* telomere sequence.
Abbreviations of restriction enzyme sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; S, *Sal*I; Sc, *Sac*I; ScII, *Sac*II; and Xh, *Xho*I

SH6173. Ten Ura$^+$ transformants were analyzed for their karyotypes by CHEF gel electrophoresis and Southern hybridization. One transformant exhibited the expected split YACs, which were 490 kb and 100 kb (Fig. 1C and Fig. 2). To verify that these two new YACs were generated by the splitting of the 590-kb YAC, Southern hybridization analysis was performed using the *TK* gene and *URA3* as probes and the probes were hybridized with the 100-kb split YACs (Fig. 2). These observations indicate that the 490-kb and 100-kb fragments harboring the amplification system originated from the 590 kb YAC.

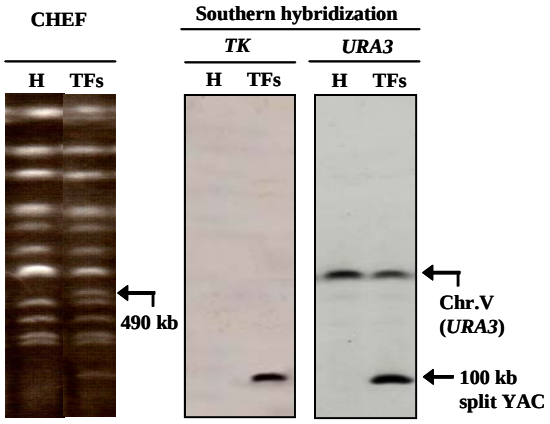

Fig. 2. Splitting of 100 kb from the right end of YAC CIC9e2. Karyotypic analysis of split-chromosomes by CHEF gel electrophoresis and Southern hybridization analysis using labeled 2.1 kb PCR product of the *TK* gene and labeled 780 bp PCR product containing the *URA3* gene as probes. The *URA3* gene was hybridized to its wild-type locus on chromosome V and to the 100 kb YAC. Lane H, *S. cerevisiae* strain SH6173; lane TFs, Ura$^+$ transformant.

To examine whether the copy number of the 100-kb split YAC containing amplification devices was amplified, the strain with split YAC was grown under various concentrations of selective reagents as inducing substances to evaluate the YAC copy number. The selective conditions for copy number amplification are strongly inhibitory for cell growth. Typically, the YAC clones required 3-6 days to reach saturation in liquid medium and a minimum of about seven generations of growth was required for significant amplification. For the amplification, about 10^7 cells were inoculated into 50 ml of amplification medium and grown for five days. After the preparation of DNA plugs, CHEF electrophoresis and Southern hybridization were performed. In YPAD medium, the 100-kb split YAC was present at about 1 copy per haploid genome (Fig. 3, lane 2). However, in the amplification medium with various amounts of selective reagents, S1/M10, S1/M50, S3/M10 and S3/M50, increases in copy number were observed (Fig. 3, lanes 3, 4, 5, and 6). The 100-kb split YAC was amplified readily to 14.1 copies/cell at 3 mg/ml sulfanilamide and 50 μg/ml methotrexate (S3/M50). The copy numbers of other sizes of split YAC clones (e.g., 150 kb) were also increased under copy num-

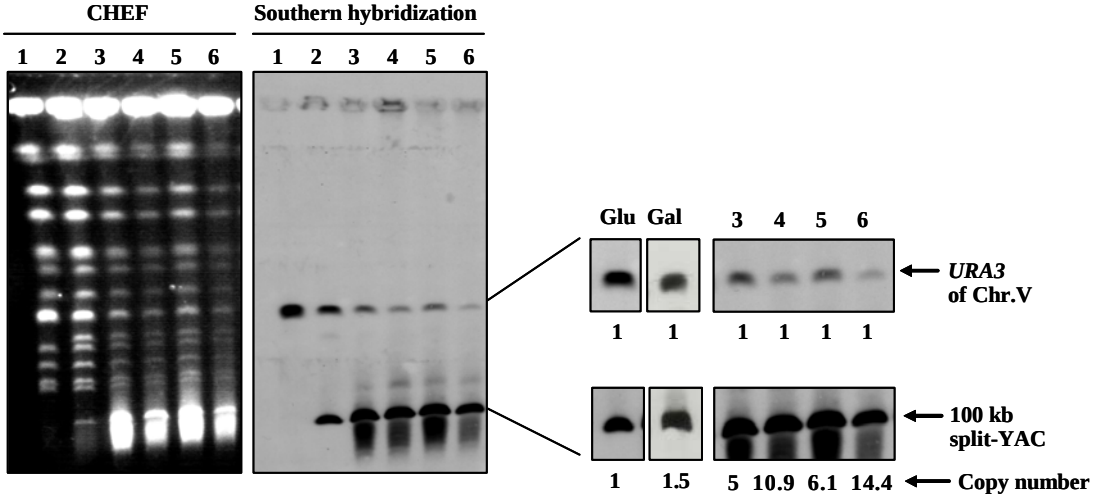

Fig. 3. CHEF electrophoresis pattern and Southern blot hybridized with a *URA3* as a probe of amplified 100 kb spit-YAC. The copy numbers of split-YAC were estimated ratio of *URA3* on split-YAC relative to a single copy gene, *URA3* on natural chromosome V. Host strain, SH6173 and strain containing 100 kb split-YAC following growth on YPAD medium (lane 1 and lane 2, respectively). Also, strain containing 100 kb split-YAC was grown on amplification medium with various amounts of S1/M10, S1/M50, S3/M10 and S3/M50 (lane 3, 4, 5 and 6, respectively). S1, S3 - 1 mg/ml and 3 mg/ml sulfanilamide; M10, M50 - 10 μg/ml and 50 μg/ml methotrexate.

ber-inducing substances (data not shown). These results demonstrate that artificial chromosomes containing a targeted region of a plant chromosome can be readily amplified, and that this system will contribute not only to the acceleration of physical mapping but also to functional analysis of eukaryotic genes.

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초록 : 복제수 증폭시스템과 염색체 분단기술을 이용한 Simultaneous YAC Manipulation-Amplification (SYMA) 시스템의 개발

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복잡한 진핵생물에서의 물리적 지도 작성이나 기능해석에 효모인공염색체(YAC)를 이용하기 위해서는 원하는 target region의 인공염색체화 및 single-copy인 YAC의 복제수를 늘이는 것이 요구된다. 본 연구에서는 YAC manipulation system에 복제수 증폭시스템(copy number amplification system)을 도입한 Simultaneous YAC Manipulation-Amplification (SYMA) system을 구축하였다. 식물염색체를 가진 YAC clone의 splitting과 증폭을 위해 conditional centromere와 thymidine kinase (TK) 유전자를 가진 pBGTK plasmid를 구축하였고, splitting fragment의 PCR을 위한 주형으로 사용하였다. 590 kb의 YAC clone은 splitting과 동시에 copy number amplification element를 가진 100 kb YAC와 490 kb YAC로 분리되었고, 100 kb YAC는 유도기질로 3 mg/ml sulfanilamide와 50 μg/ml methotrexate (S3/M50)의 첨가에 의해 14.4배로 그 복제수가 증가하였음을 확인할 수 있었다.