Simultaneous Overexpression of Integrated Genes by Copy Number Amplification of a Mini-Yeast Artificial Chromosome

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A copy number amplification system for yeast artificial chromosomes (YACs) was combined with simultaneous overexpression of genes integrated into a YAC. The chromosome VII (1,105 kb) was successfully split to 887 kb, 44 kb containing the element for copy number amplification, and a 184-kb split-YAC. The 44-kb split-mini YAC was amplified a maximum of 9-fold, and the activity of the reporter enzymes integrated into the split-mini YAC increased about 5–7-fold. These results demonstrate that the mini-YAC containing a targeted chromosome region can be readily amplified, and the specific genes in the mini-YAC could be overexpressed by increasing the copy number.

Keywords: Yeast artificial chromosome, PCR-mediated chromosome splitting, copy number amplification, simultaneous overexpression

The yeast artificial chromosome (YAC) system, which allows isolation of larger DNA fragments and easier modification of the cloned DNA than other systems, is an important tool for functional analysis and gene identification [1]. To manipulate large DNA fragments cloned into YACs, various YAC splitting methods have been developed [2–4]. Although YACs have versatile applications, they are limited as single-copy artificial chromosomes. Smith *et al.* [5] reported the incorporation of elements that enable the amplification of YAC copy number, such as a conditional centromere or the thymidine kinase (*TK*) gene. If an effective amplification system for YACs is available, the physical mapping and functional analysis of the genome will be simplified, resulting in the ability to increase the copy number of various genes on the YAC. In a previous study, we developed an effective YAC splitting method by incorporating the PCR-mediated chromosome splitting method [6, 7] and reported that the artificial chromosome containing the targeted region of a plant chromosome can be readily amplified [8]. Therefore, we attempted the overproduction of recombinant enzymes and an increase in the expression level of genes in the YAC by introducing the amplification system into a mini-YAC.

In the present study, several enzymes requiring xylan/

xylose metabolism were used as model enzymes, and a mini-YAC harboring a four-gene expression cassette was constructed. The *XYLP*, *XYLB*, *GRE3*, and *XYL2* genes encoding endoxylanase, β -xylosidase, xylose reductase, and xylitol dehydrogenase, respectively, were stably integrated into the yeast chromosome VII in a previous study (unpublished). *Saccharomyces cerevisiae* SEY2102 Δ trp/pRS-XylP, pRS-XylB, pRS-Gre3, pRS-Xyl2 strain (designated PBG2 strain), constructed by introducing the integrative plasmid with each gene (pRS-*GENE*), was used as the host strain for chromosome manipulation.

The YPD nutrient medium and synthetic complete medium for *S. cerevisiae* and cultivation methods have been described in a previous report [6], and YPDG (YPD containing 1% galactose) medium was used for galactose-inducible gene expression. To amplify the split-mini YAC, the amplification medium contained 0.67% (w/v) yeast nitrogen base; 1% (w/v) casamino acids; 3%, 4%, and 5% (w/v) galactose; 0.8 mg/ml thymidine; 3 mg/ml sulfanilamide; and 50 μ g/ml methotrexate (S3/M50). Yeast transformation was performed according to the high efficiency transformation protocol [6]. To generate each splitting fragment, pSKcLEU2 [6], pSKURA3, pSKCEN4, and pBGT were used as template plasmids for PCR. The pBGT plasmid, which

Table 1. Plasmids and primers used in this study.

Plasmids	Description	Products
pSKcLEU2	pBluescript II SK-loxP-CgLEU2 (<i>Candida glabrata</i> LEU2 gene)-loxP gene	SF-I
pSKCEN4	pBluescript II SK-CEN4 gene	SF-II
pSKURA3	pBluescript II SK-URA3 gene	SF-III
pBGT	pBluescript II SK-GAL1p/CEN4-TK gene	SF-IV
Primers	Sequences (5'-3')	Products
ADE3-1	CTCTATCGGTGCCTCTTCTG	SF-I
ADE3-2	<u>TAGTGAGGGTTAATTGCGCGCTTGGCGTAA</u> TCGATGACGGCCTTG	
ADE3-3	<u>TAGTGAGGGTTAATTGCGCGCTTGGCGTAA</u> AGCTTCCAACCAACC	SF-II
ADE3-4	TCCCAATAGTGTTCGTATTA	
PDX1-1	AAGGTGACAAGGTCCTCGAA	SF-III
PDX1-2	<u>TAGTGAGGGTTAATTGCGCGCTTGGCGTAA</u> TAGTACTGAAGCAAC	
PDX1-3	<u>TAGTGAGGGTTAATTGCGCGCTTGGCGTAA</u> TCTGCGGATGGCTTC	SF-IV
PDX1-4	GTGAGCGACCAGCAACGAGA	
SK-F	TTACGCCAAGCGCGCAATTA	All
Tr-R	CCCCAACCCCAACCCCAACCCCAACCCCAACCCCAATAATACGACTCACTATAGGG	

Underlined letters indicate overlap sequences used for the second PCR.
PCR products (splitting fragments I–IV) were amplified by PCR using these plasmids as a template and each primer set.

has components for amplifying a split-mini YAC, was constructed by inserting the *TK* gene and *GAL1p/CEN4* into the pBluescript II SK+ vector. The plasmids and oligonucleotides used are listed in Table 1. The pulsed field

gel electrophoresis (PFGE) and Southern hybridization carried out have previously been described by Kim *et al.* [6]. The enzyme activities of endoxylanase, β -xylosidase, xylose reductase, and xylitol dehydrogenase were measured

Fig. 1. Splitting position in chromosome VII of *Saccharomyces cerevisiae* strain PBG2 (A) and structure of the mini-artificial chromosome harboring the xylan metabolism system and copy number amplification system (B).
TEL: *Tetrahymena* telomeric (C₄A₂)₆ repeat sequence; CEN4: centromere of chromosome IV; GAL1p: promoter of *GAL1* gene; TK: thymidine kinase gene; CgLEU2: *LEU2* gene of *Candida glabrata*.

by each assay method [9–11].

Chromosome VII of the PBG2 strain was selected to split and manipulate because it has the *XKS1* gene encoding xylulokinase, and the xylan metabolism-related gene cluster was integrated into the *PMT6* gene position (Fig. 1A). In order to manipulate the mini-YAC from chromosome VII, two target genes (*ADE3* and *PDX1*) that do not affect cell viability or growth even when the gene is disrupted or overexpressed by splitting or copy number increase, respectively, were selected for splitting. To split the 184-kb region from the right end of chromosome VII, two splitting fragments (SF-I and SF-II) harboring each target sequence for homologous recombination were amplified. SF-I consisted of *CgLEU2*, a telomeric (5'-C₄A₂-3')₆ repeat sequence, and a target sequence. The *CgLEU2* gene used to select yeast transformants was first amplified by PCR using pSKcLEU2 as a template and SK-F and Tr-R as the forward and reverse primers, respectively. Independently, a 500-bp target sequence that corresponded to a sequence from

nucleotide positions 908,074 to 908,574 of chromosome VII was amplified by PCR using genomic DNA from the PBG2 strain as template and ADE3-1 and ADE3-2 as primers. A 30-bp overlap sequence was attached to the end of the ADE3-2 primer. The *CgLEU2* and target sequence were then used as templates and Tr-R and ADE3-1 as primers for a second PCR to generate the 2.3-kb SF-I. The other SF-II (1.5 kb) containing *CEN4*, a telomeric repeat sequence, and the target sequence (nucleotide position 908,575 to 909,075 of Chr. VII) was also amplified by two rounds of PCR using the same procedure as that for SF-I preparation, except with different template DNA and primers. The two amplified splitting fragments were purified and transformed into the PBG2 strain. Four Leu⁺ transformants were analyzed for their karyotypes by PFGE, and three transformants exhibited the expected split-YACs, 925 kb and 184 kb from manipulated chromosome VII (1,105 kb) (Fig. 2A). We selected one transformant among the strains with split-YACs and named it the R-split strain. Subsequently, we

Fig. 2. Analysis of split chromosomes by PFGE (A and B) and schematic diagram of the splitting procedures in chromosome VII by the PCR-mediated chromosome splitting method (C).

Manipulated chromosome VII of 1,105 kb in strain PBG2 was split to 925-kb and 184-kb split YACs (A). Subsequently, the 925-kb split-YAC was more split to 887 kb and a 44-kb mini-chromosome harboring the xylan degradation system (B). Lane 1: 2102Δtrp strain (Host strain); lanes 2–5: R-split strain No. 1–4; lanes 6–8: RL-split strain No. 1–3. Confirmation of integration position by PFGE and Southern hybridization (D). *XYLP*, *XYLB*, *GRE3*, and *XYL2* genes were used as probes. Lane 1: 2102Δtrp strain (Host strain); lane 2: PBG2 strain; lane 3: R-split strain; lane 4: RL-split strain.

split the 887-kb region from the left end of chromosome VII. Two splitting fragments (SF-III and SF-IV) harboring each target sequence were amplified using the same procedure used for SF-I and SF-II construction. Briefly, SF-III (2.2 kb) consisted of the *URA3* gene as a selection marker for yeast transformants, a telomeric sequence, and a target sequence (nucleotide positions 884,509 to 885,009 of Chr. VII). Another SF-IV (4 kb) consisted of *GAL1P/CEN4* (GC4), the *TK* gene, a telomeric sequence, and a target sequence (nucleotide position 885,010 to 885,510 of Chr. VII). To simultaneously introduce an amplification system with chromosome splitting, SF-III and SF-IV were transformed into the R-split strain. Two *Ura*⁺ transformants showed that the 925-kb split-YAC was successfully split into a new 887-kb split-YAC and a 44-kb split-mini YAC containing the amplification system (Figs. 2B and 2C). One transformant was called the RL-split strain. To prove that these new YACs were generated from the splitting of chromosome VII, Southern hybridization was performed using *XYLP*, *XYLB*, *GRE3*, and *XYL2* genes as probes, and the probes hybridized to the 44-kb split-mini YAC (Fig. 2D). These observations indicate that the 44-kb fragment

harboring the amplification system and the xylan metabolism system originated from the manipulated chromosome VII (Fig. 1B).

To confirm amplification of the copy number of the 44-kb split-mini YAC containing the amplification system, the RL-strain was grown with selective reagents and galactose to evaluate the YAC copy number. The principle of copy number amplification has previously been described [8]. After preparation of DNA plugs, PFGE and Southern hybridization were performed. In YPD medium, the 44-kb split-mini YAC was present at about 1.2 copies per haploid genome (Fig. 3A, lane 2). However, in amplification medium with selective reagent (S3/M50) and 3%, 4%, and 5% galactose, increases in the copy number were observed (Fig. 3A, lanes 3, 4, and 5). The 44-kb split-mini YAC was amplified readily to about 9.0 copies/cell with 5% galactose. Moreover, endoxylanase activity increased at 24.72 unit/ml in the copy number amplification strain (CA strain), although cell growth was decreased by adding the selective reagent and galactose, which induce missegregation and slow cell division (Fig. 3B). The activity of β -xylosidase, xylose reductase, and xylitol dehydrogenase was also successfully increased about 5–7-fold compared with no induction of amplification. These results demonstrate that the mini-YAC containing the targeted chromosome region can be readily amplified, and the specific genes in the mini-YAC could be overexpressed by increasing the copy number. This is the first report of simultaneous overexpression of reporter genes by amplifying the copy number of split-mini YAC. This system will provide not only simultaneous overproduction of several enzymes, but also novel strain breeding by allowing transplantation of mini-YACs into industrial strains.

Fig. 3. Confirmation of the copy number amplification by PFGE and Southern hybridization. (A) *GRE3* gene used as probe. The copy numbers of the split-mini YAC were estimated as the ratio of *GRE3* on split-mini YAC relative to a single-copy gene, *GRE3*, on natural chromosome VIII. The intensity of hybridization signals with the *GRE3* gene probe was measured using the scion image program. Lane 1: PBG2 strain; lane 2: RL-split strain; lanes 3–5: RL-split strain induced into amplification medium containing 3%, 4% and 5% galactose, respectively. (B) Comparison of cell growth (OD₆₀₀) and enzyme activity (unit/ml) in RL-split and copy number amplification (CA) strains.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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