Simultaneous and Sequential Integration by Cre/*loxP* Site-Specific Recombination in *Saccharomyces cerevisiae*

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A Cre/*loxP*- δ -integration system was developed to allow sequential and simultaneous integration of a multiple gene expression cassette in *Saccharomyces cerevisiae*. To allow repeated integrations, the reusable *Candida glabrata* MARKER (CgMARKER) carrying *loxP* sequences was used, and the integrated CgMARKER was efficiently removed by inducing Cre recombinase. The XYLP and XYLB genes encoding endoxylanase and β -xylosidase, respectively, were used as model genes for xylan metabolism in this system, and the copy number of these genes was increased to 15.8 and 16.9 copies/cell, respectively, by repeated integration. This integration system is a promising approach for the easy construction of yeast strains with enhanced metabolic pathways through multicopy gene expression.

Keywords: δ -Sequence integration, Cre/*loxP* system, *Candida glabrata* MARKER, simultaneous multiple integration

Saccharomyces cerevisiae plays a key role in industries ranging from pharmaceutical production to ethanol and biomass production and is an important industrial microorganism. In all of these applications, the ability to introduce and maintain stable foreign genes is extremely important because large-scale production of foreign genes in recombinant yeast often uses complex medium. High-copy-number plasmids derived from YE_p are often mitotically unstable, and selective conditions are required for the maintenance of these plasmids. However, YE_p vectors may be unstable at high expression even in selective conditions [1, 2]. Therefore, direct integration of a target gene into the host chromosome is an effective method for the stable expression of foreign genes in *S. cerevisiae* [3]. However, traditional integration methods using mitotically stable vectors such as YI_p have limitations regarding the number of genes that can be inserted, low copy number, and regulation of the integration process. To increase the integration copies of a target gene, repetitive chromosomal DNA sequences such as δ -sequences [4–7] and rDNA [8, 9] have been used. In general, δ -sequences were more versatile than rDNA sequences, and high-level expression of cloned genes using a sequential δ -integration method was reported

[3]. For repeated and sequential integration, incorporation of a reusable selective marker and δ -sequence-mediated integration are necessary. In the original δ -integration method, the *hisG-URA3-hisG* cassette was used as a reusable selective marker, but it was impossible to simultaneously integrate multiple genes. In a previous study, we developed a PCR-mediated chromosome splitting method combined [10] with the Cre/*loxP* site-specific recombination system for marker rescue and repeatedly split chromosomes [11, 12]. Using this system, we attempted introduction of a reusable selective marker system for the sequential and/or simultaneous integration of multiple genes by δ -sequence-mediated integration.

In the present study, two enzymes (endoxylanase and β -xylosidase) required for degradation of xylan were used as model enzymes, and integrative plasmids harboring the *Candida glabrata* MARKER (CgMARKER) carrying the *loxP* sequences and δ -sequence were constructed. *S. cerevisiae* SEY2102 Δ trp (*MAT α ura3-52 leu2-3, 112 his4-519 suc2- Δ 9 trp1::KanMX*) was used as the host strain, and the XYLP and XYLB genes encoding endoxylanase and β -xylosidase, respectively, were stably integrated into the yeast chromosome by δ -sequence-mediated integration. The YPD nutrient

medium and synthetic complete (SC) medium for *S. cerevisiae* and cultivation methods have been described in a previous report [11]. YPDG (YPD containing 1% galactose) medium was used for galactose-inducible gene expression. 5-Fluoroorotic acid (5-FOA) medium was used for removal of the pSH47 plasmid containing the *URA3* selective marker [11]. The methods for yeast transformation, pulsed-field gel electrophoresis (PFGE), Southern hybridization, and thin-layer chromatography were previously described by Kim *et al.* [13, 14]. The enzyme activities of endoxylanase and β -xylosidase were measured by respective assay methods [15, 16].

The pRS δ -T and pRS δ -H plasmids were constructed for integration of the *XYLP* and *XYLB* genes, respectively, into the δ -sequence of the yeast chromosome. We used the *YFLWdelta4* sequence (332 bp) as the δ -sequence because it shares high homology (>90%) with other δ -sequences [17]. The *YFLWdelta4* sequence was cloned into pRS306 (a Yip vector), resulting in the pRS δ plasmid. For repeated integration by introduction of a reusable selective marker, the *loxP*-*CgTRP1*-*loxP* (1.2 kb) and *loxP*-*CgHIS3*-*loxP* (1.7 kb),

Candida glabrata TRP1, and *HIS3* cassette carrying the *loxP* sequences (34 bp) were cloned into the pRS δ plasmid, resulting in the pRS δ -T (6 kb) and pRS δ -H (6.5 kb) plasmids, respectively. The *CgTRP1* and *CgHIS3* genes (72% and 64% nucleotide sequence identity with the corresponding *S. cerevisiae* genes, respectively) were used to avoid undesirable recombination between the integrative plasmids and the marker loci within the *S. cerevisiae* genome. Finally, the *XYLP* and *XYLB* gene expression cassettes, *GAL10p*-*MF α* *s.s*-*XYLP*-*Gal7t* and *GAL10p*-*MF α* *s.s*-*XYLB*-*Gal7t* from the pGMF α -*XylP* [18] and pGMF α -*XylB* plasmids, respectively, were inserted into the pRS δ -T and pRS δ -H plasmids, resulting in the pRS δ -*XylP* and pRS δ -*XylB* plasmids.

pRS δ -*XylP* was linearized by digestion with *XhoI*, which is a unique restriction site within the δ -sequence, and introduced into the SEY2102 Δ *trp* strain. Transformants expressing endoxylanase were selected on tryptophan-deficient SC plates, and one transformant that showed endoxylanase activity by Congo-red staining was designated the δ -P strain (Fig. 1A). The endoxylanase was efficiently secreted into the medium by mating factor α signal sequence

Fig. 1. Procedure for δ -sequence-mediated integration of the *XYLP* and *XYLB* gene expression cassette and construction of yeast strains with endoxylanase and β -xylosidase activities.

The endoxylanase activity was determined by Congo-red assay. H and T indicate the SEY2102 Δ *trp* host strain and δ -P strain, respectively. MF α *s.s*: mating factor α signal sequence of *S. cerevisiae*; *CgTRP1*: *TRP1* gene of *Candida glabrata*; *loxP*: ATAACCTTCGTATAATGTATGCTATACGAAGTTAT (for sequential integration).

Fig. 2. Analysis of the copy numbers of endoxylanase and β-xylosidase genes using RT-PCR (A) and comparison of cell growth (OD₆₀₀), and endoxylanase and β-xylosidase activities in each strain (B).

Total RNA was isolated from each transformant, and cDNA was synthesized from total RNA by RT-PCR. The *XYLP*, *XYLB*, and *ACT1* (internal control) genes were amplified by PCR using each cDNA as template, and the products were visualized by agarose gel electrophoresis. Lane 1: δ-P strain; lane 2: δ-PP strain; lane 3: δ-PPB strain; lane 4: δ-PPBB strain.

(MFas.s). For sequential integration, the *CgTRP1* selective marker of the δ-P strain was removed by introducing the pSH47 plasmid (*GAL1p-CRE-CYC1t*). The Cre recombinase was expressed in YPDG medium for induction of galactose, and a δ-P Δtrp cell that had lost the *CgTRP1* gene by excisional recombination between the two direct *loxP* repeats was selected. The pSH47 plasmid harboring the *URA3* gene as a selective marker was also cured by selection and spreading onto YPD medium containing 5-FOA (Fig. 1B). The goal of employing the *loxP*-*CgMARKER*-*loxP* selection cassette was to allow sequential and simultaneous integrations of the same or different genes via δ-integration. Therefore, the pRSδ-XylP plasmid was retransformed into the δ-P Δtrp strain by the same procedure, and the δ-PP strain was also constructed (Fig. 1C). Subsequently, pRSδ-XylB was linearized by digestion with *XhoI* and introduced into the δ-PP strain. Transformants with β-xylosidase activity were selected on histidine-deficient SC plates, and

transformants showing β-xylosidase activity by two rounds of δ-integration were designated the δ-PPB and δ-PPBB strains (Fig. 1C). Analysis of cell samples before and after *CgMARKER* curing by Southern hybridization and auxotrophic phenotype analysis confirmed that the *CgTRP1* and *CgHIS3* markers were efficiently removed (>80%) from the integrated chromosomes by Cre recombinase (data not shown). To determine the copy number of integrated *XYLP* and *XYLB* genes, each strain was grown in YPDG medium, and the copy numbers were compared by RT-PCR and signal intensity analysis [18]. In the δ-PP and δ-PPBB strains, a 2-fold increase in copy number was observed compared with the δ-P and δ-PPB strains, respectively, by additional integration. Finally, the copy number of integrated *XYLP* and *XYLB* genes was 15.8 and 16.9 copies/cell, respectively, in the δ-PPBB strain (Fig. 2A). The activities of endoxylanase and β-xylosidase in the δ-PP and δ-PPBB strains were also increased approximately 1.6–1.7 fold compared with the δ-P and δ-PPB strains, respectively, (Fig. 2B), and the endoxylanase activity was stably maintained despite repeated marker rescue, cultivation, and transformation. The *XYLP* genes were intensively integrated in chromosomes VIII and V in the δ-PP strain (Fig. 3A, lane 2), and the *XYLB* genes tended to be intensively integrated into chromosomes VIII, V, and XIV (Fig. 3B). The increased copy number was presumed to be caused by integration targeted to a new location, as described in Lee and Da Silva [19]. The integrated *XYLP* and *XYLB* genes were stably maintained in chromosomes in non-selective medium over 60 generations compared with the gene introduced as an episomal type, and 98% of endoxylanase activity was maintained in the δ-PPBB strain (Fig. 3C), indicating that expression of the cloned gene did not significantly affect the structural stability. Furthermore, the endoxylanase and β-xylosidase produced in the integrants efficiently hydrolyzed xylan to xylose and xylooligosaccharides (xylobiose and xylotriose) (Fig. 4). For more efficient employment of the *CgMARKER*-δ-integration system, the pRSδ-XylP and pRSδ-XylB plasmids were simultaneously introduced into the SEY2102Δtrp strain, and transformants were selected on tryptophan and histidine-deficient SC plates. A transformant showing endoxylanase and β-xylosidase activities was selected (δ-PB strain), and the enzyme activity was not significantly different from that of sequential integration (one round) (Fig. 2B). Moreover, the double selective markers were synchronously removed (>75%) by inducing Cre recombinase (data not shown). These results demonstrated that the Cre/*loxP*-mediated δ-integration system was more versatile for

Fig. 3. Confirmation of the integration position by PFGE and Southern hybridization.

Gal10p (A) and *XYLB* genes (B) were used as probes. Lane H: SEY2102Δtrp; lane 1: δ-P strain; lane 2: δ-PP strain; lane 3: δ-PPB strain; lane 4: δ-PPBB strain. Comparison of the mitotic stability and residual endoxylanase activity in the SEY2102Δtrp/pGMFa-Xylp (episomal type) and δ-PPBB (integrative type) strains (C). These strains were cultivated for 60 generations in YPDG in a 50-ml baffled flask culture.

sequential and simultaneous integration of a gene expression cassette of two or more genes compared with the original δ-integration system.

Fig. 4. Comparison of degradation products of xylan by thin-layer chromatography.

Each transformant was cultivated in YPDG medium for 48 h, and 1% xylan (birchwood) was hydrolyzed by enzymes produced by each transformant. Lane S: standard mixture (xylose, xylobiose, and xylotriose); lane 1: δ-P strain; lane 2: δ-PP strain; lane 3: δ-PPB strain; lane 4: δ-PPBB strain.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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