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Simultaneous and sequential integration by Cre/loxP site-specific recombination in \textit{Saccharomyces cerevisiae}

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Running Title; SIMULTANEOUS AND SEQUENTIAL INTEGRATION BY CRE/LOXP SYSTEM
The 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 *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 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 YEp are often mitotically unstable, and selective conditions are required for the maintenance of these plasmids. However, YEp 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 stable expression of foreign genes in S. cerevisiae [3]. However, traditional integration methods using mitotically stable vectors such as YIp 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 was used the hisG-URA3-hisG cassette as a reusable selective marker, but, it was impossible to simultaneously integrate multiple genes. In a previous study, we developed the PCR-mediated Chromosome Splitting (PCS) 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 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 CgMARKER carrying the loxP sequences and δ-sequence were constructed. *S. cerevisiae* SEY2102∆trp (*MATα ura3-52 leu2-3, 112 his4-519 suc2-Δ9 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. 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 pSH47 plasmid containing the *URA3* selective marker [11]. The methods for yeast transformation, pulsed field gel electrophoresis (PFGE), Southern hybridization, and thin layer chromatography (TLC) 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].

pRSδ-T and pRSδ-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 pRSδ 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 pRSδ plasmid, resulting in pRSδ-T (6 kb) and pRSδ-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-MFa s.s-XYLP-Gal7t* and *GAL10p-MFa s.s-XYLB-Gal7t* from
pGMFα-XylP [18] and pGMFα-XylB plasmids, respectively, were inserted into pRSδ-T and pRSδ-H plasmids, resulting in pRSδ-XylP and pRSδ-XylB plasmids.

pRSδ-XylP was linearized by digestion with XhoI, which is a unique restriction site within the δ-sequence, and introduced into the SEY2102Δtrp 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 medium by mating factor α signal sequence (MFαs.s). For sequential integration, the CgTRP1 selective marker of the δ-P strain was removed by introducing 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 integrated chromosomes by Cre recombinase (data not shown). To determine the copy number of integrated XYLP and XYLB genes, each strain was grown with 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 two-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 activity of endoxylanase and β-xylosidase in the δ-PP and δ-PPBB strain was also increased approximately 1.6–1.7 fold compared with the δ-P and δ-PPB strain, respectively, (Fig. 2B), and the endoxylanase activity was stably maintained despite repeated marker rescue, cultivation, and transformation. The XYLp genes were intensively integrated in chromosome 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 et al [19]. The integrated XYLp and XYLb genes were stably maintained in chromosomes in non-selective medium over 60 generations compared with gene introduced as 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 structural stability. Furthermore, the endoxylanase and β-xylosidase produced in the integrants efficiently hydrolyzed xylan to xylose and xylooligosaccharides (xylodiose 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 activity was selected (δ-PB strain), and the enzyme activity was not significantly different from that of sequential integration (one round) (Fig. 2B). Also, 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 sequential and simultaneous integration of a gene expression cassette of two or more genes compared with the original δ-integration system.
Conflict of interest

The authors declare that they have no competing interest.

REFERENCES


15. Lee LH, Kim DY, Han MK, Oh HW, Ham SJ, Park DS, Bae KS, Sok DE, Shin DH,
    Son KH, Park HY. 2009. Characterization of an extracellular xylanase from *Bacillus*
    sp. HY-20, a bacterium in the gut of *Apis mellifera*. *Kor. J. Microbiol.* 45: 332-338.
    and the nucleotide sequence of a *Bacillus* sp. KK-1 β-xylosidase gene. *J. Microbiol. Biotechnol.* 8:
    28-33.
    S. 2003. Repeated chromosome splitting targeted to delta sequences in *Saccharomyces*
    2013. Efficient secretory expression of recombinant endoxylanase from *Bacillus* sp.
19. Lee FW, Da Silva NA. 1997. Sequential delta-integration for the regulated insertion of

**Figure legends**

**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 activity. The
    endoxylanase activity was determined by congo-red assay. H and T indicate SEY2102Δtrp host
    strain and δ-P strain, respectively. MFα s.s: mating factor α signal sequence of *S. cerevisiae*,
    *CgTRP1*: *TRP1* gene of *C. glabrata*, loxP: ATAACTTCGTATAATGTGCTATACGAAGTTAT (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 (OD$_{600}$), endoxylanase, and β-xylosidase activity 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.

Fig. 3. Confirmation of 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 mitotic stability and residual endoxylanase activity in SEY2102Δtrp/pGMF-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.

Fig. 4. Comparison of degradation products of xylan by thin layer chromatography (TLC). Each transformant was cultivated in YPDG medium for 48 hr, 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.
(A) Integration of endoxylanase expression cassette at the δ-sequence: transformation of linearized pRSδ-XylP plasmid into SEY2102Δtrp

(B) CgTRP1 gene marker rescue by introducing pSH47(GAL1p-CRE-CYC1t)

Remove pSH47 plasmid on 5-FOA medium after CgTRP1 marker rescue

(C) 2nd introduction of pRSδ-XylP plasmid by repeated δ-integration

1st introduction of pRSδ-XylB plasmid by repeated δ-integration

2nd introduction of pRSδ-XylB plasmid by repeated δ-integration

Fig. 1. Procedure for
(A) | $XYLP$ | $XYLB$ |
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<td>7.9</td>
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$\rightarrow$ Copy numbers

$\rightarrow$ $ACTI$

(B)

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<th>Strains</th>
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<th>Endoxylanase activity (unit/ml)</th>
<th>$\beta$-xylosidase activity (unit/ml)</th>
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Fig. 2. Analysis of the copy numbers of endoxylanase and
Fig. 3. Confirmation of integration position by PFGE and Southern hybridization. Gal10p (A) and XYL genes (B) were used as probes. Lane H: SEY2102

<table>
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