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Development of Reusable Split *URA3*-Marked Knockout Vectors for *Saccharomyces cerevisiae*

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Abstract Two knockout vectors, in which the truncated *Kluyveromyces lactis URA3* gene is flanked by a direct repeat, were developed for *Saccharomyces cerevisiae*. Each vector was designed to harbor 5'- and 3'-end homology regions for integration. Two knockout fragments were devised to integrate into the correct locus in a complementary manner to disrupt a gene of interest and concomitantly to make functional *Kl URA3* for transformant selection. The use of dual complementary knockout cassettes was expected to dramatically reduce integration into unwanted loci in the genome. The knockout system developed in this study was successfully used for disruption of the *GAL1* gene in *S. cerevisiae*.

Key words: *Saccharomyces cerevisiae*, knockout, *URA3*

Numerous molecular biological tools have rapidly evolved for budding yeast, *Saccharomyces cerevisiae*, which has been one of the most widely used eukaryotic workhorses in biotechnology [4, 8, 10, 12]. In particular, gene disruption techniques became essential for analysis of ORF (open reading frame) functions, which often require tedious polymerase chain reactions (PCRs) using several sets of primers [2, 5]. Although homologous recombination at the correct locus occurs at a relatively high rate in the yeast, knockout cassettes with a short homology region or tail might integrate into unwanted loci in the genome, which inevitably results in complicated and laborious selection procedures [2, 7]. A PCR-based disruption method that significantly increased knockout efficiency using long homology regions for integration was reported [9].

This study was performed to develop two complementary knockout vectors for *S. cerevisiae* to improve the integration frequency of a knockout cassette into the correct locus in the genome. The truncated *Kluyveromyces lactis URA3* (*Kl URA3*) gene and each 5'- and 3'-end homology regions were incorporated into two separate vectors. The two separate knockout cassettes were designed to integrate into the correct locus in the chromosome in a complementary manner, to make the defective marker *Kl URA3* intact only after correct homologous recombination and, hence, to select true knockout transformants (Ura^+) at a high rate. The applicability of the system developed was tested to disrupt the *GAL1* gene encoding galactokinase in *S. cerevisiae*.

Escherichia coli DH5α was used for DNA manipulations. *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1 can1 his3 ura3-52*) was used as the host for the *GAL1* gene disruption [3]. A mutant *S. cerevisiae* (*gal1Δ::kanMX4*) with BY4742 genetic background (*MAT*a *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) was purchased from Open Biosystems (Huntsville, AL, U.S.A.) and used as the control. Plasmid pWJ1077, a kind gift from Dr. Rothstein (Columbia University, New York, U.S.A.), harbors the *Kl URA3* gene flanked by a direct repeat (142 bp), which was originated from the 5'-untranslated region of the *Kl URA3* [9].

LB medium (1% NaCl, 1% tryptone, 0.5% yeast extract) supplemented with ampicilln (50 mg/l) was used for bacterial cultivation. YEPD (1% yeast extract, 2% peptone, 2% glucose) and YEPG (1% yeast extract, 2% peptone, 2% galactose) plates were used for the phenotype assay. An SC (synthetic complete) lacking uracil was supplemented with glucose (2%) for transformant selection. Yeast transformations were performed as described elsewhere [11].

PCR amplifications were performed using GeneAmp 2400 (Applied Biosystems, Foster City, CA, U.S.A.). Primers listed in Table 1 and polymerases were purchased from Bioneer (Daejeon, Korea). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, U.S.A.).

Two different kinds of knockout vectors, pWAL100 and pWBR100, were made from pWJ1077. Primers Fa and Ra were used to amplify a fragment flanking direct repeat and

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Table 1. PCR primers used in this study.

Name	Sequences (5'-3')
Fa	AAAGAGCTCGGATCCACTAGTGCGGCCGCCATGGTCGTGATTCTGGGTAGAAGATCGG
Ra	AAAGACAGAAGAGCAACAATGATGATATCTGATCCACCA
Fb	AAAGAGCTCCGCAGAATGGTCTGATATCACCAA
Rb	AAACAGCTGCCATGGCGGCCGCACTAGTGGATCCTACCGGGCCCGTGTCACCATGA
f-ALGB	AAAGGATCCCCCTCCGACGGAAGACTCTCCT
r-ALGN	AAACCATGGTATAGTTTTTTCTCCTTGACGTTAAAGT
f-BRGB	AAAGGATCCGTATACTTCTTTTTTTTACTTTGTTCAGA
r-BRGN	AAACCATGGGGGTTCTCGTAGAGTCCCCGGA
r-AKUD	TTGGTGACCTCTTGCGCACCTTG
f-BKUD	TCATGACCCCAGGTGTAGGTTTAG

truncated *Kl URA3* (1-699) using pWJ1077 as the template. This PCR product was treated with SapI/SacI and cloned into pWJ1077 digested with the same restriction enzymes to make pWAL100. To construct plasmid pWBR100, pWJ1077 was digested with SapI, blunt-ended, and treated again with SacI/PvuII enzymes to clone a PCR fragment containing the direct repeat and truncated *Kl URA3* (360-803), which was amplified from pWJ1077 using primers Fb and Rb. As results, the knockout vectors pWAL100 and pWBR100 harbor only one repeat sequence down- or upstream of the truncated *Kl URA3* gene (Fig. 1).

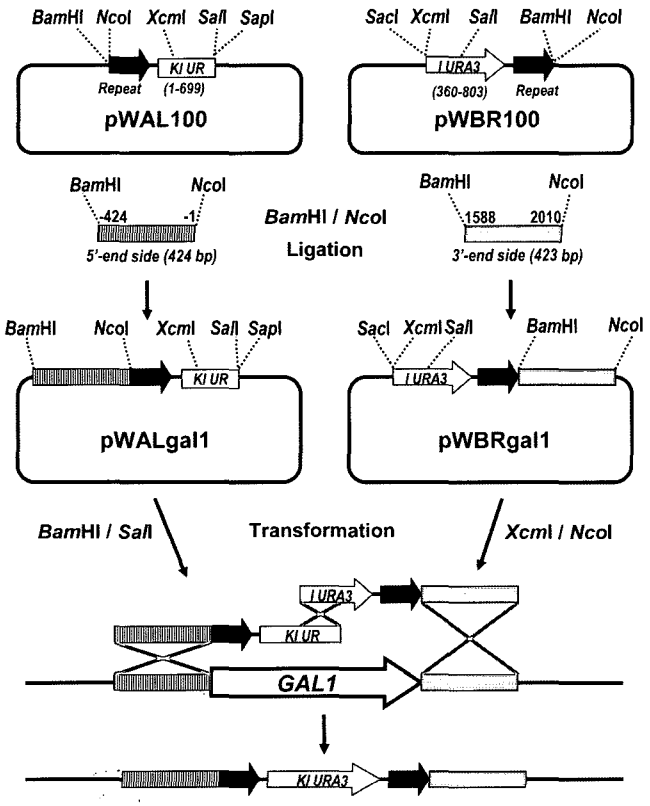

Fig. 1. Schematic representation of knockout vector construction and *GAL1* gene disruption.

To prepare 5'- and 3'-homology regions, approximately 420 bp upstream and downstream fragments of the *GAL1* gene were amplified using two sets of primers: f-ALGB and f-ALGN for the 5'-homology region and r-BRGB and r-BRGN for the 3'-homology region. Each PCR fragment was introduced into the BamHI/NcoI sites of pWAL100 and pWBR100 to construct pWALgal1 and pWBRgal1, respectively. The two separate knockout cassettes were released by digesting pWALgal1 with BamHI/SalI and pWBRgal1 with XcmI/NcoI, and the expected-size products were transformed into *S. cerevisiae* 2805. A schematic representation of vector construction and *GAL1* gene disruption is shown in Fig. 1.

S. cerevisiae 2805 strains transformed with two complementary knockout cassettes were selected on a SC-ura$^-$ plate. Diagnostic PCR were performed using the primers listed in Table 1 to validate integration of the selection module. Twenty randomly chosen Ura$^+$ colonies were "true" transformants (data not shown). Moreover, transformation of only one of two knockout cassettes did not give Ura$^+$ colonies, indicating that any single knockout cassette alone does not work as a selection marker. As depicted in Fig. 2, the PCR products of expected size and growth phenotypes were obtained.

PCR-based methods allow single-step deletion of chromosomal genes [5, 9, 15]. These methods rely on the amplification by PCR of a selectable marker using two primers with tail sequences that are homologous to the desired target gene into which the cassette is to be integrated, followed by transformation and homologous recombination. The advantages of this strategy are speed and general applicability; practically any desired ORF can be deleted, since the entire *S. cerevisiae* genome sequence is known and publicly available. However, PCR-based methods have drawbacks; i.e., in some cases if not all, a knockout cassette is integrated into an unwanted locus in the genome, which renders tedious selection procedures. Transformation efficiency with the PCR products having small homology regions at both ends is inferior to that with DNA having long homology. One of the plausible methods to reduce the

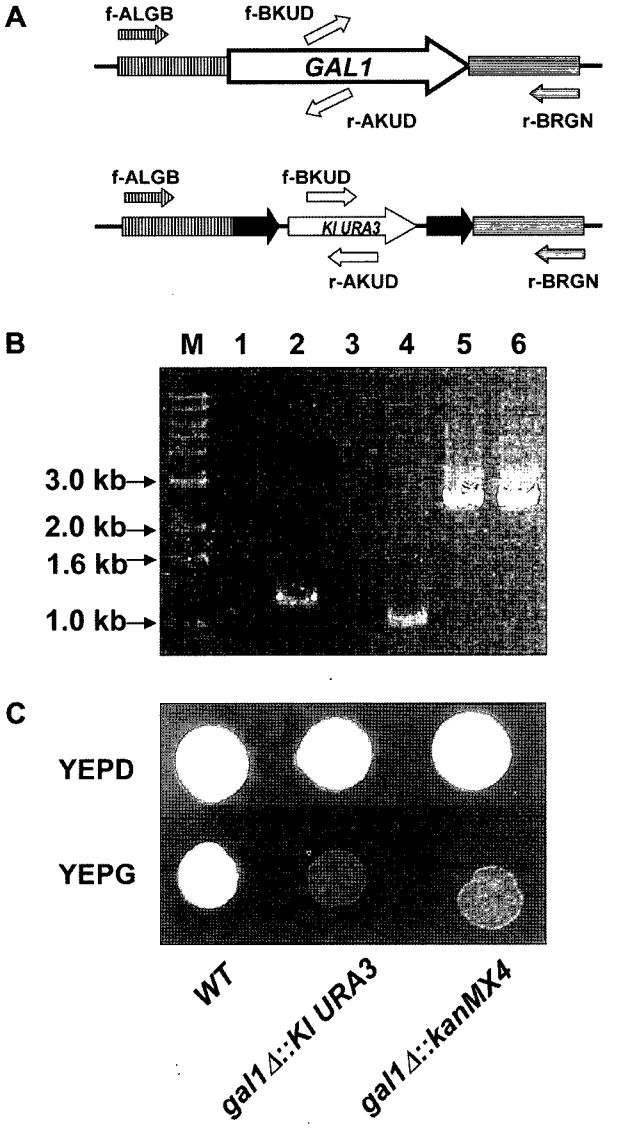

Fig. 2. Schematic illustration of diagnostic PCR (**A**), agarose gel electrophoresis of PCR products (**B**), and plate assay (**C**) to confirm integration of the knockout cassette into the *GAL1* locus in the *S. cerevisiae* 2805 genome.

Primer sets used were f-ALGB/r-AKUD (lanes 1, 2), f-BKUD/r-BRGN (lanes 3, 4), and f-ALGB/r-BRGN (lanes 5, 6). Lanes 1, 3, and 5 were obtained from wild-type (WT). Lanes 2, 4, and 6 were products from mutant strain (*gal1Δ::Kl URA3*). For plate assay shown in panel **C**, cells with the indicated genotypes were grown in YEPD, washed twice with water, and spotted on each plate at concentrations of 1×10^7. A mutant strain (*gal1Δ::kanMX4*) with BY4742 strain background was used as the control. Photos were taken after two days at 30°C.

frequency of unwanted integration might be the use of tail or homology sequences with "proper length" [2].

As the number of marker genes is limited, efficient procedures for "marker rescue" is very important for functional analysis of multiple genes. Counter-selection is a powerful technique in yeast for repeated gene disruptions via excision of a selection module [3, 6, 14]. The most popular one is the loss of *URA3*, which can be selected on a 5'-FOA plate.

Another type of a marker rescue system is based on the use of a site-specific recombinase and its target sites [1, 13]. Specifically, the Cre-*lox*P recombination system of bacteriophage P1 has been shown to mediate efficient recombination between *loxP* sites flanking a marker gene in *S. cerevisiae*, resulting in excision of the marker gene [13]. However, this method uses the Cre-expression vector that requires additional transformation with another marker besides the marker for knockout and, furthermore, the Cre-expression vector should be cured before physiological studies. Accordingly, spontaneous excision of a marker flanked by a direct repeat is highly desirable.

In this study, we developed two knockout vectors for *S. cerevisiae*. Since neither vector has a functional *Kl URA3*, the two independent knockout cassettes released by restriction digestion should be simultaneously and complementally integrated into a correct locus to make an intact and functional *Kl URA3* for selection. In other words, two truncated *Kl URA3* fragments recombine as a functional form only after a correct homologous integration event. Conclusively, it was expected that the frequency of unwanted integration of a knockout cassette could be significantly reduced by using the "split *URA3*-marked" knockout cassettes. The applicability of this system was tested by deleting the *GAL1* gene, and every Ura^+ colony was found to be a true knockout transformant to give the predicted Gal^- phenotype as shown in Fig. 2C. Other vectors harboring a split marker such as *kanMX4* and *natMX6* can be developed to integrate a genetic module of interest at the correct locus in the genome at high frequency.

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