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Research Article 研究报告

A doxycycline-inducible gene deletion and marker recycling toolkit in *Candida albicans*

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Abstract: [Objective] To construct a fast, convenient and economical DNA manipulating toolkit for gene deletion and marker recycling in *Candida albicans*. **[Methods]** By using *Exo* III-mediated ligase-independent cloning strategy, *loxP* sites were inserted into the flanking regions of heterologous selection markers *CmLEU2*, *CdHIS1*, and *CdARG4*, yielding the templates for marker cassettes amplification. The Tet-on promoter was assembled using a *rTetR* element which was codon-optimized and chemically synthesized. Codon-optimized recombinase Cre was placed downstream of the Tet-on promoter. Then this cassette was inserted into the CDS region of selection markers *CdHIS1* and *CdARG4*, generating vectors for marker recycling. **[Results]** We constructed three *loxP*-flanked marker gene-containing vectors for gene deletion in *C. albicans*, and two vectors for marker recycling, containing the recombinase gene Cre under the control of the Tet-on promoter. **[Conclusion]** We successfully constructed an inducible gene deletion and marker recycling vector system in *C. albicans*, which was practically applied in gene deletion strain constructions. This system is helpful to construct single and multiple gene deletion strain constructions.

Keywords: *Candida albicans*, gene deletion, Tet-on, marker recycling, Cre, *loxP*

Candida albicans is one of the most important fungal pathogens of humans. It causes systemic candidiasis, which is life-threatening for patients with deficient immunity[1]. Its pathogenicity is tightly correlated with its virulence and host-pathogen interactions. To study the molecular mechanisms of its pathogenicity, it is very important to construct single or multiple genes deletion mutants for exploring their functional roles.

In recent years, many molecular tools for gene deletion and marker recycling were reported for *Candida albicans*[2–4]. *URA3* blaster method was the first and important one for gene deletion in *Candida albicans*[5]. This method has been applied and adapted in many published papers and genetic toolkits for *Candida albicans*[6–9]. However, the

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URA3 marker was reported to have a negative effect on its hyphal development and virulence[10–11]. Its flanking repeats of hisG may also affect the expression of *URA3* and subsequent multiple gene disruptions[12–13]. In addition, 5-FOA which was used for *URA3* excision may induce chromosome alterations in *Candida albicans*[14], complicating the phenotypes and genotypes of mutant strains. To avoid the use of *URA3*, new reference strains (SN serial strains) and heterologous auxotrophic markers (i.e. *Candida maltosa LEU2*, *Candida dubliniensis HIS1* and *Candida dubliniensis ARG4*) were applied and preferred in this field[15].

Marker recycling is important for multiple gene deletions. In *C. albicans*, *FLP-FRT* system was adapted to excise *URA3* or *SAT1* selection marker, which was called *URA* flipper or *SAT1* flipper [16–17]. The Cre-*loxP* system was also applied with traditional selection markers and an inducible promoter *MET3*p for marker loop-out, called Cre-*loxP* flipper and Clox flipper[6,18]. The famous genetic manipulation tool, CRISPR/Cas9 system has also been developed for *C. albicans*[19]. Based on CRISPR, an efficient gene deletion and marker recycling system was constructed[20–21].

In this study, we reported a fast, convenient and cost-effective strategy for gene deletion and marker recycling in *C. albicans*. This system uses *loxP*-flanked markers with short homology regions to delete genes. Cre was controlled by the Tet-on promoter which could be effectively induced to express by doxycycline (Dox, a derivative of tetracycline). The Tet-on promoter is widely used in mammalian cells and was also introduced into the *Candida* field for tetracycline-inducible gene expression[22]. After induction, all selection markers will be excised and be ready for next genetic manipulation.

1 Materials and Methods

1.1 Strains and growth conditions

Candida albicans strains used in this study are listed in Table 1. SN152 was kindly provided by Suzanne M. Noble[15]. The other strains were constructed following the illustration of Figure 3. Simply, the *loxP-CmLEU2-loxP* cassette was amplified from pCPC48 with primers which were designed following the introduction of Figure 1-C. This cassette was introduced into SN152 to delete one copy of the target gene. *loxP-CdHIS1-loxP* was amplified from pCPC49 with the same primers and was introduced into the heterozygote to delete the second copy of the target gene, generating the homozygous null mutant. Then HACreH cassette was amplified from pCPC51 and was introduced into the mutant to perform marker excision by Dox induction. After excision, only one *loxP* site was left at the original locus for each copy.

Table 1. *Candida albicans* strains used in this study

Strain	Parent	Description	Source
SN152	RM1000#2	*arg4- leu2- his1-*	[15]
CPS18	SN152	*sif2::CmLEU2/sif2::CdHIS1*	This study
CPS50	SN152	*gcn5::loxP-CmLEU2-loxP/gcn5::loxP-CdHIS1-loxP*	This study
CPS55	CPS50	*gcn5:: loxP/gcn5:: loxP*	This study
CPS52	SN152	*set3::loxP-CmLEU2-loxP/set3::loxP-CdHIS1-loxP*	This study
CPS56	CPS52	*set3:: loxP/ set3:: loxP*	This study
CPS146	SN152	*flo8::loxP-CmLEU2-loxP/flo8::loxP-CdHIS1-loxP*	This study
CPS147	CPS146	*flo8:: loxP/ flo8:: loxP*	This study

All *Candida* strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or SC (synthetic complete, 0.67% yeast nitrogen base with ammonium sulfate, 2% glucose, appropriate amino acid mix) medium. Doxycycline hydrochloride was bought from Sangon Biotech (China) and was added at a final concentration of 50 μg/mL. The *Escherichia coli* strain DH5α was used for molecular cloning and was selected in LB medium supplemented with 100 μg/mL of ampicillin or 50 μg/mL of kanamycin.

1.2 Plasmid construction and PCR conditions

pSN40, pSN52 and pSN69, which contains *CmLEU2*, *CdHIS1* and *CdARG4* respectively, were kindly provided by Suzanne M. Noble[15]. *CmLEU2*, *CdHIS1* and *CdARG4* were amplified from pSN40/52/69 using *loxP*-F and *loxP*-R primers (Table 2, *loxP* sites was shown in bold and italic). The three *loxP*-flanked markers were then introduced into the backbone fragment amplified from pUC19 (TransGen, Beijing) using primers pUC18+762s and pUC18+2629a, via *Exo* III-mediated ligation-independent cloning (LIC)[23], generating pCPC48, pCPC49, and pCPC50 respectively.

The rTetR (reverse tetracycline repressor) element was chemically synthesized by QinlanBio, China. It was codon-optimized using OPTIMIZER[24] according to the codon usage table of *C. albicans* (http://www.kazusa.or.jp/codon/), encoding the same protein of Tet-on 3G of pCMV-Tet3G Vector of ClonTech, TaKaRa, Japan. The rTetR element was amplified by primers TetR-F and TetR-R. The GAL4AD element was amplified by primers GAL4AD-F and cartTA-R. These two elements formed a fusion protein called cartTA (*Candida albicans* reverse tetracycline-controlled trans-activator). The tetracycline responsive element P_{tet} was amplified using primers Ptet-F and OP4-R. The P_{tet} element and GAL4AD were all amplified from pCaUme6-3[25]. The caCre gene was amplified from pCAD[6] using primers Cre-F and Cre-R. The *ADH1* promoter was amplified using primers *ADH1*-735s and *ADH1*p-R. The *ACT1* terminator was amplified using primers Tact1+1765s and Tact1+2070a. The *HIS1* marker was amplified using primers *HIS1*-446s and *HIS1*+1149a. The *ARG4* marker was amplified using primers *ARG4*-331s and *ARG4*+1635a. The *ADH1* promoter, the *ACT1* terminator, *HIS1* and *ARG4* were all amplified from genomic DNA of the wildtype *Candida albicans* strain SC5314. Finally, the *ADH1* promoter, *cartTA*, the *ACT1* terminator, *ARG4*, P_{tet}, and CaCre were sequentially assembled with the backbone amplified from pSN52 using primers *CdHIS1*-F and *CdHIS1*-R via LIC, generating pCPC51. Similarly, the *ADH1* promoter, *cartTA*, the *ACT1* terminator, *HIS1*, P_{tet} and CaCre were sequentially assembled with the backbone amplified from pSN69 using primers *CdARG4*-Fand *CdARG4*-R via LIC, generating pCPC52.

All PCR amplification used the KOD-plus enzyme (Toyobo, Japan) following the manufacturer's instruction. The integrity of all DNA cassettes and correct cloning of all constructs were verified by sequencing (MajorBio, Shanghai).

The complete reference sequences of these plasmids are available with GenBank Accession numbers: pCPC48 (MG874799), pCPC49 (MG874800), pCPC50 (MG874801), pCPC51 (MG874802), pCPC52 (MG874803).

1.3 Primers

Primers used in this study were all synthesized by Sangon, Biotech (China) with HAP purification and are listed in Table 2. *loxP* sites were shown in italic and bold.

1.4 *C. albicans* transformation and mutant validation

C. albicans are directly transformed with PCR products containing *loxP*-marker-*loxP* with 78 bp flanking homology region from target genes without purification. The transformation was performed by following an improved protocol[26]. All mutants were validated via diagnostic PCR using gene-specific primers and universal primers VP9 & VP10 for *CmLEU2*, VP11 & VP12 for *CdHIS1*, VP13 & VP14 for *CdARG4* (Table 2).

Table 2. PCR primers used for plasmid construction, cassettes amplification and verification

Primer	Sequence (5′ → 3′)
loxP-F	AGAAA***ATAACTTCGTATAGCATACATTATACGAAGTTAT***AGCTCGGATCCACTAGTAAC
loxP-R	TTACC***ATAACTTCGTATAATGTATGCTATACGAAGTTAT***ACCAGTGTGATGGATATCTG
pUC18+762s	ACGAAGTTATGGTAATACGGTTATCCACAGAAT
pUC18+2629a	ACGAAGTTATTTTCTTAGACGTCAGGTGGCACT
TetR-F	AATACAAAAACAATTATGTCAAGATTAGATAAATCAAAAGTTATTAAT
TetR-R	ACCAAACAAAGTCGAACCACCTGATTCACATTTTAATTGT
GAL4AD-F	TCGACTTTGTTTGGTGGCGCCAATTTTAATCAAAGT
cartTA-R	GTAGCGATGCACGGTTTACTCTTTTTTTGGGTTTGGTGG
Ptet-F	CAGTCAGTCAGTCAGGCTTGGACAGCATCAGCAGTAG
OP4-R	ATTGACAATGATTACCTATTTATATTTGTATGTGTGTAGGAGT
Cre-F	AGGTAATCATTGTCAATGTCTAACTTGTTGACCGTTCA
Cre-R	GTAGCGATGCACGGTTGTTACGAATCAATGGCACTA
ADH1-735s	ACAGTCTGCCAGACTGCCAGCGAGAAAGAGGAGTAT
*ADH1*p-R	AATTGTTTTTGTATTTGTTGTTGTTGTTGTATGACAAT
Tact1+1765s	ACCGTGCATCGCTACCATTGTTCACCACAAATGTTTCT
Tact1+2070a	AGTCTGGCAGACTGTAGATATAGAAATGCCTTGGG
HIS1-446s	ACAGTCTGCCAGACTTGTATGGTAATCCAAATGGG
HIS1+1149a	CTGACTGACTGACTGATGGTGCTCATGGCTACGC
ARG4-331s	ACAGTCTGCCAGACTCAATTTATGACATATTGACCGAC
ARG4+1635a	ACTGACTGACTGACTGCATTTGCAAGTATGGTTAGG
CdARG4-F	ACCGTGCATCGCTACAAATTGTCGGGAATTGACCA
CdARG4-R	AGTCTGGCAGACTGTTGACCAAACACTCTGCCACT
CdHIS1-F	ACCGTGCATCGCTACGACCATGAAAGCTGCTGGAT
CdHIS1-R	AGTCTGGCAGACTGTCAATTGGCAAGTTTGTGGAA
VP9	AGCTTCACCAAACCCAGCTA
VP10	CCGGTTTACTTGGATCTTCG
VP11	CATTTCACACCCAGCTCGTA
VP12	ACGACGGCTGATTTGTCTTT
VP13	AACACACCATCGAAAAAGTCG
VP14	CAACCTTTCAAACGATGCAA
AHCreA-F	CTCATAAAGAAATTGATGTATTAATGCCTGGT
AHCreA-R	TGGTCTGGATGGAGAGAAATATAGTGACAAAGAT
HACreH-F	CCGGTACCTGGAGGACGAAGAGACCGAAGTTAGT
HACreH-R	CGAGAATGCCCATTGATTTTAAAGGTGTGT

TcxP sites were shown in italic and bold.

2 Results

2.1 Components and strategy for marker cassettes amplification

Our goal was to create gene deletion cassettes with short homology regions fast and cost-effectively. Therefore, we chose heterologous auxotrophic markers which could reduce the mistargeting effect of homologous markers[15]. Besides, marker recycling was also required. We chose the Cre-*loxP* system for its high efficiency[6].

As shown in Figure 1-A, *CmLEU2*, *CdHIS1* and *CdARG4* were amplified from pSN40, pSN52 and pSN69 respectively with primers containing 34 nt *loxP* sites and were then inserted into the same backbone of pUC19 via *Exo* III-mediated cloning (LIC), generating ready-to-use *loxP*-marker-*loxP* cassettes. This DNA assembly method is convenient and efficient because only 15 bp homology is necessary for sequential multiple fragment assembling. The backbone of pUC19 here contained only the replication origin and ampicillin resistance gene. Since the three markers were introduced into the same backbone, universal primers could be used for marker cassette amplification.

These cassettes could be amplified using F1 and R1 primers in the Round 1 PCR. Because F1/R1 primer sets comprised a common binding region (20 nt, Figure 1-C) and target gene-specific region (39 nt), the PCR #1 product was flanked by 39 bp homology regions. F2/R2 primers sets comprised a 20 nt region homologous to F1/R1 and additional 39 nt homology regions. PCR #1 product could be used as templates for PCR #2 directly and generated final marker cassettes flanked by 78 bp homology regions (Figure 1-B). This successive PCR strategy avoids the need for long primer (>60 nt) synthesis and generates enough PCR products for transformation directly.

For marker integration verification, we predesigned universal verification primers, i.e. VP9&10 were used for *CmLEU2* integration verification, VP11&12 for *CdHIS1* and VP13&14 for *CdARG4* (Figure 1-A). Diagnostic PCR could be easily performed using gene-specific primers and these validation primers.

2.2 Components and concepts for marker recycling

To excise markers in the cells efficiently, Cre must be controlled by an inducible promoter. *MET3*p promoter was used in previous reports[6]. However, the special medium was necessary to induce or inhibit the expression of Cre. To simplify this system, we chose tet-on promoter to control Cre expression since it could control gene expression efficiently and tightly in *C. albicans*[22].

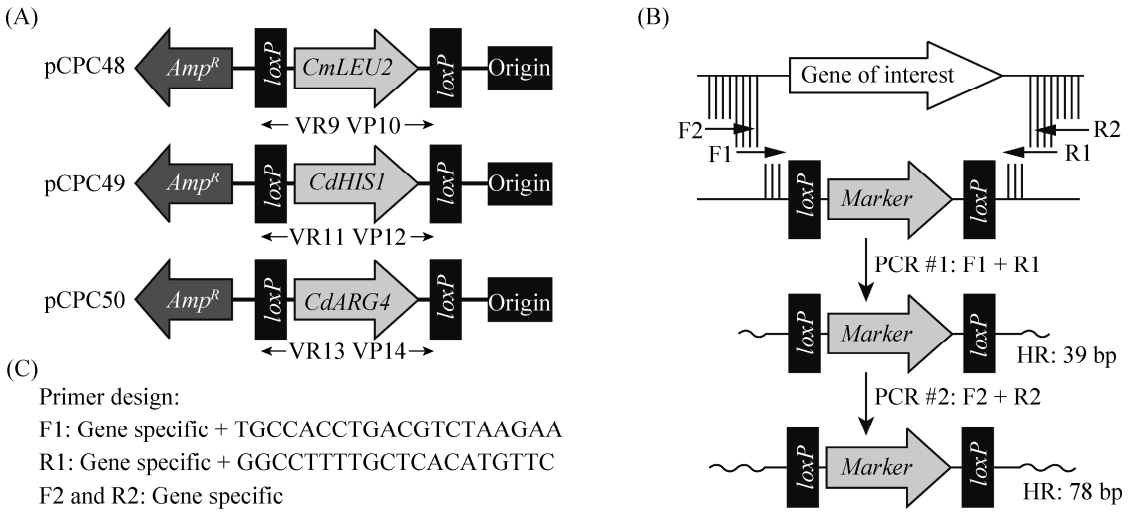

Figure 1. Strategy for *loxP*-flanked markers amplification. A: Three heterologous auxotrophic markers flanked by two 34 bp *loxP* sequences. Arrows: primers. Not to scale. *Cm*: *C. maltosa*; *Cd*: *C. dubliniensis*. Amp^R: ampicillin resistance. Origin: plasmid replication origin in *E. coli*. B: Schematic depiction of marker-*loxP* cassettes amplification. HR: homology region. Primer binding sites are indicated by vertical lines. F1/2: forward primer. R1/2: reverse primer. C: Simple primer design for amplification of marker cassettes.

The Tet-on 3G promoter system is recommended by the Clontech company since it has lower background activity and higher sensitivity. To adopt this, we performed codon optimization of the core element of Tet-on 3G promoter, i.e. rTetR (reverse tetracycline repressor), according to the codon usage database (http://www.kazusa.or.jp/codon/), obtaining the *carTetR* element. Then we fused it to the codon-optimized GAL4AD element amplified from pCaUme6-3[25], generating the fusion gene cartTA (*Candida albicans* reverse tet transactivator). The constitutive promoter, *ADH1*p, was used to control the expression of this fusion gene. The *ACT1* terminator was placed downstream of the fusion gene to stop translation efficiently. Classical selection marker, *ARG4*, was placed upstream of the tetracycline-responsive promoter P_{tet}, which was also amplified from pCaUme6-3. The codon-optimized recombinase caCre was amplified from pCAD[6] and placed downstream of the P_{tet} promoter. Then these fragments were introduced into the coding region of pSN52 via one-step reaction of LIC, generating pCPC51 (Figure 2-A). Similarly, *HIS1* was used to replace *ARG4* and these fragments were introduced into the coding region of pSN69 via LIC, generating pCPC52 (Figure 2-A).

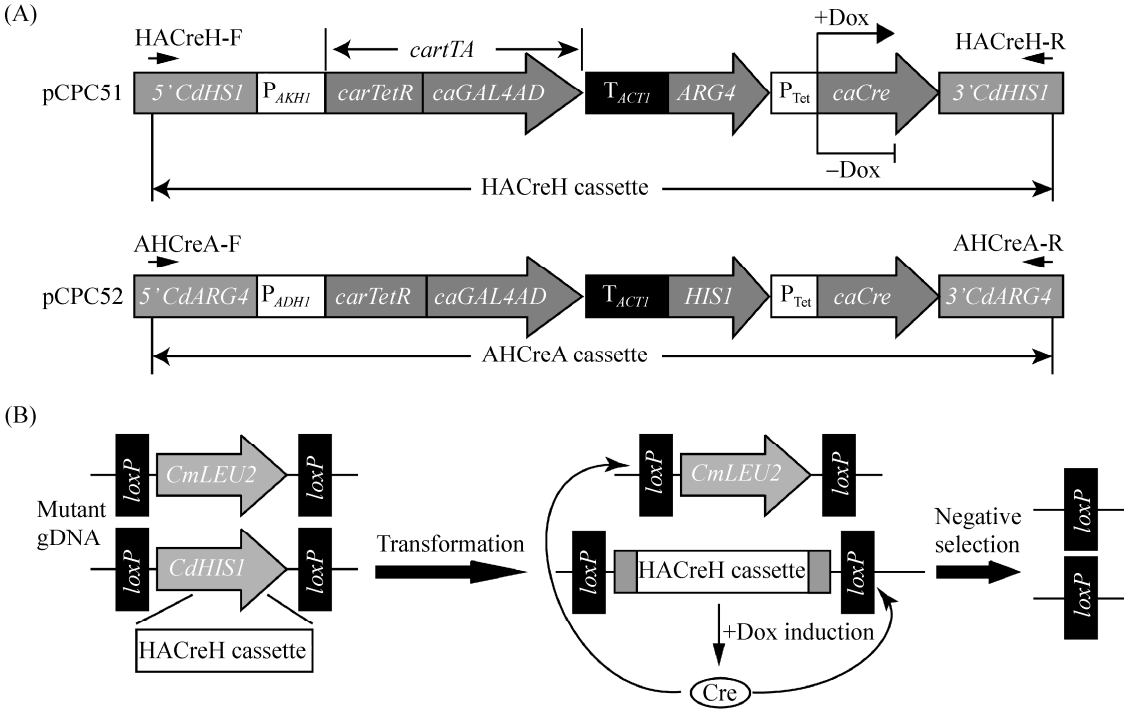

Figure 2. Concepts for marker recycling through Dox-induced marker excision in *C. albicans*. A: Components of the Tet-on promoter controlled Cre cassettes. Short arrows: primers. ca: codon-optimized for *C. albicans*. Dox: doxycycline. Not to scale. P: promoter. T: terminator. ca: *C. albicans* codon-optimized. rtTA: reverse tetracycline-controlled trans-activator protein. AD: activation domain. Cre: *loxP* site-specific recombinase. HACreH-F/R and AHCreA-F/R are primers used to amplify HACreH or AHCreA cassettes as indicated by long arrows. The backbones of vectors were omitted. B: Schematic illustration of Cre-mediated marker excision. Alleles of target genes are deleted using PCR-generated *CmLEU2/CdHIS1* cassettes. HACreH cassette containing a Tet-on promoter controlled caCre element and *ARG4* marker is then introduced into the coding region of *CdHIS1*. Then Cre is induced by adding Dox and catalyzes site-specific recombination between *loxP* sites and leave a single *loxP* site at the same locus. Clones with no auxotrophic markers could be obtained by negative selection.

Since the *ADH1* promoter is constitutive in *C. albicans*, the fusion protein cartTA is expressed constitutively. In the absence of Dox, the fusion protein could not bind to the P_{tet} region, resulting in the silence of the recombinase Cre. In the presence of Dox, the fusion protein could interact with Dox and then binds to the P_{tet} region steadily, sustaining a very high expression of the recombinase Cre. Once the recombinase Cre was efficiently expressed, a recombination event can occur between the *loxP* sites, leaving one copy of *loxP* site.

For convenience, the core cassette of pCPC51, i.e. 5′ *CdHIS1*-P_{ADH1}-cartTA-T_{ACT1}-*ARG4*-P_{tet}- caCre-3′ *CdHIS1*, was named HACreH. Likewise, the core cassette of pCPC52, 5′ *CdARG4*-P_{ADH1}-cartTA-T_{ACT1}-*HIS1*-P_{tet}-caCre-3′ *CdARG4*, was named AHCreA. Predesigned primer sets HACreH-F/R (for pCPC51) or AHCreA-F/R (for pCPC52) could amplify the HACreH or AHCreA cassettes efficiently and the PCR products are ready-to-use for marker recycling.

When markers are required to be excised in a gene deletion mutant (e.g. *CmLEU2* and *CdHIS1* mutant), HACreH cassette was introduced into mutant cells via transformation and *CdHIS1* would be then disrupted. Subsequently, transformants were treated with Dox to induce Cre expression. Finally, Cre could catalyze site-specific recombination between *loxP* sites, resulting in marker excision and only one *loxP* site left in the target gene locus (Figure 2-B).

2.3 A detailed protocol for this approach

Here we describe a detailed protocol for target gene deletion and marker excisions (Figure 3). This protocol was used many times in practice and generated sufficient positive transformants each time. The first step, to delete a gene, *loxP-CmLEU2-loxP*, and *loxP-CdHIS1-loxP* cassettes with 78 bp homology regions are amplified via two rounds of PCR. PCR products are transformed into competent cells directly via two rounds of transformation to generate

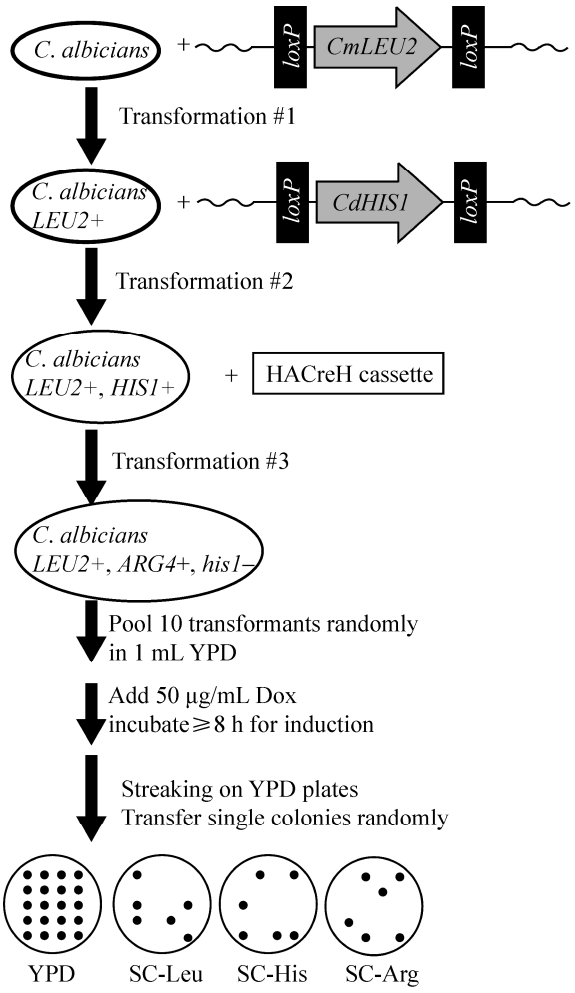

Figure 3. A detailed protocol for gene deletion and marker excision. Conventional PCR amplified *CmLEU2-loxP* and *CdHIS1-loxP* cassettes with 78 bp homology regions are introduced into the locus of the target gene via two rounds of transformation to generate a gene deletion mutant of *LEU2+* and *HIS1+*. For marker excision, PCR amplified HACreH cassette is introduced into this mutant via the third transformation to generate a mutant of *LEU2+*, *ARG4+* and *his1-*. Randomly, about 10 transformants are pooled in 1 mL YPD containing 50 μg/mL Dox and incubate at 30 °C for at least 8 hours. Then streak the mixture on YPD plates for single colonies. Transfer single colonies randomly to perform negative screening on YPD and SC-Leu/His/Arg plates. The clones only grow on YPD plates are positive ones. SC: synthetic complete medium. Leu: leucine. His: histidine. Arg: arginine.

a gene deletion mutant with the genotype of *LEU2*+, *HIS1*+, which could grow on SC medium without leucine and histidine. The second step, to excise all markers, PCR amplified HACreH cassette is transformed into this mutant to generate a mutant with the genotype of *LEU2*+, *ARG4*+, *his1*-, which could grow on SC medium without leucine and arginine. Transformants are selected on SC plates without arginine. Pick out 10 transformants randomly, pool them in 1 mL YPD containing 50 μg/mL Dox and incubate at 30 °C for at least 8 hours with gentle shaking. Then streak the mixture on 1–2 YPD plates and incubate at 30 °C for single colonies. Pick out 20–40 single colonies randomly and replicate them on YPD and SC-Leu/His/Arg plates. The clones will only grow on YPD plates are positive ones. According to our practice, 60%–90% single colonies are positive clones.

2.4 Gene deletions using the Tet-on promoter controlled Cre/loxP system

To validate the functionality of the Tet-on promoter controlled Cre/*loxP* system, we used this system to delete several genes that play roles in the hyphal development of *C. albicans* in SN152 strain[15]. *SET3* and *SIF2* are two genes that coding components of histone deacetylase complex Set3C. Null mutants of these two genes were reported to have a phenotype of increased hyphal growth. By using *CmLEU2-loxP* and *CdHIS1-loxP* cassettes, we deleted *SET3* gene in *C. albicans* (i.e. CPS52) and performed marker excision, generating CPS56. *SIF2* gene was deleted by markers without *loxP* sites (i.e. CPS18). Then we checked the phenotypes of the two null mutants. *set3* and *sif2* mutants (CPS56 and CPS18) grew wrinkled colonies on YPD plates at 37 °C (Figure 4-A). In liquid YPD, these two mutants

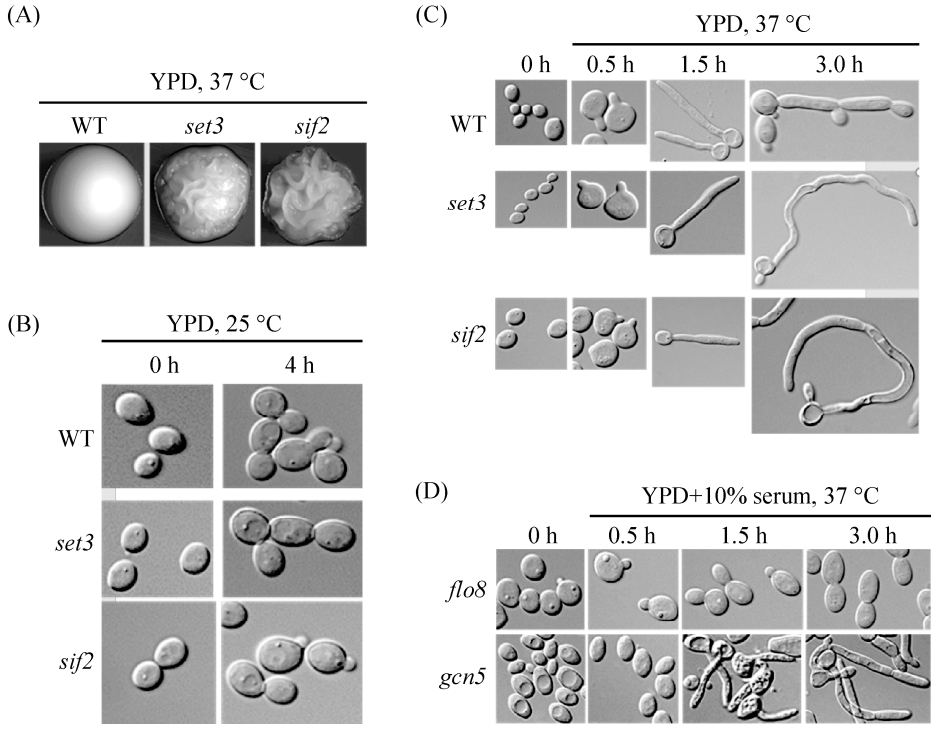

Figure 4. Gene deletion mutants using the $P_{Tet\text{-}on}$-Cre/*loxP* system. A: *set3* and *sif2* deletion mutants were plated on YPD plates and incubated at 37 °C. B: *set3* and *sif2* deletion mutants were inoculated in liquid YPD and incubated at 25 °C. C: *set3* and *sif2* deletion mutants were inoculated in liquid YPD and incubated at 37 °C. D: *flo8* and *gcn5* deletion mutants were inoculated in YPD+10% serum and incubated at 37 °C. Photos were taken at indicated time points.

grew true hyphae at 37 °C while wild-type cells grew pseudohyphae (Figure 4-B). Under YPD, 25 °C condition, these two null mutants showed no differences between them and wildtype cells (Figure 4-C). This phenotype was insistent with previous reports[27].

In addition, we also constructed another two null mutants and performed marker excision, i.e. *flo8* (CPS146 and CPS147) and *gcn5* (CPS50 and CPS55). Flo8 was essential for hyphal development and Gcn5 played important roles in hyphal elongation[28–29]. Under hyphal induction condition (YPD+10% serum, 37 °C), *flo8* mutant failed to initiate hyphal growth and *gcn5* mutant failed to form long hypha (Figure 4-D). These results showed that target genes could be deleted efficiently by using these *loxP*-flanked marker cassettes.

3 Discussion

C. albicans is an important fungal pathogen of humans and it is very important to study the molecular mechanisms of its pathogenicity. Identifying and characterizing relevant gene functions will improve our understanding about this pathogen. So multiple markers and marker recycling tools are required to construct multiple genes deletion mutants.

In recent twenty years, many genetic tools are developed for *C. albicans*. Many of them were based on *URA3* blaster method. As the *URA3* marker and 5-FOA were found to have disadvantages, *URA3*-independent strains and markers were preferable. For marker recycling, FLP/FRT system, Cre/*loxP* system and CRISPR/Cas9 system were all adapted in *C. albicans*.

In this study, we have built a system using the Tet-on promoter controlled Cre/*loxP* system to delete genes and excise markers. Three heterologous markers were flanked with *loxP* sites and 78 bp gene specific homology region could be generated after two rounds of conventional PCR (i.e. successive PCR). Heterologous auxotrophic markers, instead of conventional homologous markers, is used in this system to increase the gene targeting efficiency and to avoid the impact of *URA3* on hyphal development and virulence of *C. albicans*.

There are several benefits of this system in comparison to previously established ones. Firstly, Tet-on promoter, instead of *MET3*p, is used to control the expression of Cre, which means there is no need to change special medium for induction. Secondly, considering the price differences between common primers (<60 nt) and long primers (>60 nt), this method is significant cost-effective. In addition, this method needs no further purification steps of PCR products, which could be used in transformation directly. This successive PCR strategy is more reliable and robust than fusion PCR and yields enough positive transformants because 78 bp homology regions are sufficient for homologous genomic integration in *C. albicans*.

For marker recycling, we constructed two $P_{Tet\text{-}on}$-Cre cassettes targeting *CdHIS1* or *CdARG4*. To induce Cre expression, we only need adding Dox to the common growth medium. There is no need for primer design and special medium preparation. Besides, low-grade doxycycline which is very cheap is sufficient to generate enough positive clones. The efficiency of marker recycling would increase if change YPD to SC-Arg or SC-His medium for Dox induction since untransformed cells will not grow in the latter medium.

Using this system, we have constructed many gene deletion mutants efficiently. In summary, this system described in this study provides a simple, fast, convenient and cost-effective way to construct gene deletion mutant and excise markers in *C. albicans*.

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一种强力霉素诱导型白念珠菌基因敲除与筛选标记再循环工具包

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摘要：【目的】在白念珠菌中建立一个快捷方便经济的基因敲除与筛选标记再循环的 DNA 操作系统。【方法】通过 *Exo* III 介导的不依赖于连接酶的克隆策略，在异源筛选标记基因 *CmLEU2*、*CdHIS1* 和 *CdARG4* 基因的两侧分别插入了 *loxP* 位点，成为筛选标记基因盒扩增的模板。全基因合成了经过白念珠菌密码子优化的 *rTetR* 元件，并组装成 Tet-on 启动子。将密码子优化的重组酶 Cre 基因置于该启动子控制下。然后将他们插入筛选标记基因 *CdHIS1* 和 *CdARG4* 的 CDS 区域，形成筛选标记基因再循环载体。【结果】构建了 3 个用于白念珠菌基因敲除的侧翼含有 *loxP* 位点的筛选标记基因载体，以及 2 个含有 Tet-on 启动子控制的 Cre 酶的载体用于筛选标记基因的再循环。【结论】成功构建了一个白念珠菌中可诱导的基因敲除和筛选标记再循环的载体系统并成功应用于多个基因缺失株构建。这个系统有助于快速构建白念珠菌的单基因和多基因敲除菌株。

关键词：白念珠菌，基因敲除，Tet-on，筛选标记再循环，Cre，*loxP*

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