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Efficient vector systems for economical and rapid epitope-tagging and overexpression in *Candida albicans*

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ABSTRACT

Candida albicans is an opportunistic pathogenic fungus which causes superficial and systemic infections in immunocompromised patients. It is important to characterize the roles of genes involved in its pathogenesis, virulence, and drug resistance. Several genetic manipulation toolkits have been developed for gene function research in *C. albicans*. Here, we describe efficient vector systems that allow economical and rapid C-terminal and N-terminal epitope-tagging, inducible and constitutive promoter replacements, and ectopic gene overexpression in *C. albicans*. These systems use modularized genetic elements (conventional and non-conventional selection markers, epitope tags and promoters) and universal primers. These advantages should greatly reduce laboratory work and costs of strain construction for *C. albicans*.

1. Introduction

Candida albicans is a pathogenic fungus and is one of the most important pathogens for humans. It causes superficial and invasive fungal infections (IFIs) in immunocompromised patients at a high frequency. Since 1997, IFI-associated mortality has remained stable, of which *C. albicans* remains the predominant cause and accounts for over half of all cases in the United States (Perlroth et al., 2007). To study the molecular mechanism of the pathogenesis, virulence and drug resistance of *C. albicans*, it is important to understand the roles of genes involved in these aspects. Therefore, fast and cost-effective genetic toolkits for mutant strain construction are required for *C. albicans* research.

In recent years, several molecular tools for gene manipulation have been developed for *C. albicans* (Gerami-Nejad et al., 2009; Lai et al., 2011; Lavoie et al., 2008; Milne et al., 2011; Motaung et al., 2015; Schaub et al., 2006; Xu et al., 2014), including vectors for gene disruption, inducible gene expression, epitope-tagging, gene overexpression and ectopic reintegration. PCR-based generation of cassettes was preferred in these systems since short homology regions are sufficient to mediate homologous recombination in *C. albicans* (Gola et al., 2003). Conventionally, the *URA3* marker was used for transformant screening in *C. albicans* (Fonzi and Irwin, 1993) and has since been widely used for genetic manipulation in this organism (Bryce Wilson et al., 2000; Dennison et al., 2005; Gola et al., 2003; Wilson et al., 1999). However, it has been reported that the *URA3* marker had a negative effect on hyphal development and virulence (Brand et al., 2004; Lay et al., 1998), complicating the phenotypic analysis of mutant strains. To circumvent this, new reference strains and heterologous auxotrophic markers, i.e. *Candida maltosa LEU2* (*CmLEU2*), *Candida dubliniensis HIS1* (*CdHIS1*) and *ARG4* (*CdARG4*), were developed (Noble and Johnson, 2005). For controlled gene expression, doxycycline-inducible Tet-on and Tet-off systems were also adapted for *C. albicans* (Lai et al., 2011; Nakayama et al., 2000; Park and Morschhäuser, 2005).

Here, we report efficient vector systems for rapid and economical gene manipulation in *C. albicans*. These systems contain: 1) C-terminal tagging vectors comprising 13Myc, 3HA, GFP and *loxP*-flanked markers; 2) Tet-off promoter vectors; 3) N-terminal tagging vectors containing the constitutive promoter P_{ADH1} and epitope tags; 4) gene reintegration vectors targeting the *ADE2* (ADEnine requiring 2) gene locus. These vectors were modularized, and universal primers were designed accordingly. PCR-based amplification of cassettes and primers of standard length (< 60 bp) are adopted. By using this system, we constructed tens of verified *C. albicans* strains rapidly (Chang et al., 2015; Wang et al., 2013). We believe this system is fast, highly efficient and cost-effective, which should be helpful to the study of gene functions in *C. albicans*.

2. Materials and methods

2.1. Strains and growth conditions

The *Escherichia coli* strain DH5α was used for plasmid construction.

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Cells were grown in LB medium supplemented with 100 μg/mL of ampicillin and incubated at 37 °C. *C. albicans* strains used in this study are listed in Table S1. *Candida* strains were routinely grown on YPD medium (1% yeast extract, 2% peptone, 2% glucose) or on synthetic complete dropout medium (0.67% yeast nitrogen base with ammonium sulfate without dextrose or amino acids, 2% glucose, 2% agar, appropriate amino acid mix) for selection of prototrophic strains at 25 °C or 30 °C. YPD plus 10% bovine serum medium was used for hyphal induction (Lane et al., 2001). Doxycycline hydrochloride was bought from Sangon Biotech (Shanghai, China).

2.2. Plasmid constructions

Vectors generated in this study are listed with key features in Table S2. Generally, *CmLEU2*, *CdHIS1,* and *CdARG4* markers were respectively amplified from pSN40, pSN52 and pSN69 with *loxP*-F and *loxP*-R primers (Noble and Johnson, 2005). *URA3, HIS1,* and *ARG4* markers were amplified from the genomic DNA of *C. albicans* wild-type strain SC5314, a clinical isolate. A codon-optimized 13Myc epitope was amplified from pPR671 (Cao et al., 2006). The 3HA tag was amplified from plasmid ADH-3HA (Su et al., 2009). The GFP element was amplified from pHL471 (Hazan et al., 2002). A codon-optimized TetR element was chemically synthesized by QinlanBio (Shanghai, China), according to the codon usage table of *C. albicans* (http://www.kazusa.or.jp/codon/). The TetR element encodes the 1-207 amino acid region of Tet-Off Advanced of pTet-Off-Advanced Vector from TaKaRa Bio (Shiga, Japan). The tetracycline-responsive element P_{tet} promoter and GAL4AD were amplified from pCaUme6-3 (Zeidler et al., 2009). The *ADH1* promoter and terminator, the *ACT1* terminator, the *ADE2* gene, and other DNA sequences were all amplified from the genomic DNA of the wild-type strain SC5314. A 1.8-kb fragment from pUC19, containing the AmpR gene and the replication origin, served as the backbone for all vectors. All PCR amplifications used KOD-plus high-fidelity polymerase of ToYoBo Biotech (Osaka, Japan). All elements for each vector were inserted into the modified pUC19 backbone in one-step assembly reaction via *Exo* III-mediated ligation-independent cloning (LIC) (Li and Evans, 1997). All core cassettes for transformation in *Candida* were sequenced to verify their correctness. The sequences and annotations of all vectors are available with GenBank accession numbers (Table S2). In addition, the vectors are also available from the authors.

2.3. Primers

Primers used in this study were synthesized by Sangon Biotech (Shanghai, China) and were purified with the resin-based HAP (High-Affinity Purification) method. Universal primers and the gene-specific primer design strategy are listed in Table S3.

2.4. C. albicans transformation and mutant validation

C. albicans were directly transformed with PCR products without further purification. All transformations were performed with the modified lithium acetate transformation method (Walther et al., 2003). All mutants were validated via diagnostic PCR using gene-specific primers and universal primers (Table S3).

2.5. Co-immunoprecipitation and western blot analysis

Cells were incubated at 30 °C for 2 days and then diluted into fresh YPD (yeast cell growth) or YPD + 10% bovine serum medium (hyphal cell induction) and were harvested at indicated time points. Cells were washed with sterile water once and then were subjected to cell lysis using the FastPrep®-24 System (MP Biomedicals, Ohio, USA). Protein extraction and immunoprecipitation were performed as described previously (Lu et al., 2008). Generally, about 10 mg of total protein was subjected to immunoprecipitation using 2 μg anti-Myc antibody (Santa Cruz, California, USA) and 60 μL of IgG agarose bead slurry (Roche, Basel, Switzerland). Immunoblotting analysis was carried out according to the standard laboratory protocol. Proteins were separated by 8% SDS–PAGE and transferred to a Hybond® PVDF membrane (GE Healthcare, Boston, USA). After blocking in 5% skim-milk powder in 1× TBST (Tris-buffered saline/0.05% Tween 20), anti-Myc, anti-HA (Santa Cruz), anti-GFP (Santa Cruz), or anti-H3 antibody (Merck Millipore, Massachusetts, USA) were used for probing Myc-, HA- or GFP-tagged proteins or the histone H3, which were then detected using the secondary antibody goat anti-mouse IgG-HRP (Santa Cruz) and the Pierce™ ECL system (ThermoFisher Scientific, Massachusetts, USA).

2.6. Fluorescent microscopy

A Carl Zeiss Axioplan2 microscope with a 100× oil objective was used for all microscopy. Cells were harvested and washed once with water, followed by washing with ice-cold 50% ethanol solution once. Resuspended cells in sterile water were further analyzed by microscopy. The nuclei were stained with DAPI (4′, 6-diamidino-2-phenylindole).

3. Results

3.1. C-terminal tagging vectors

Since appropriate expression level is extremely important for protein function, it is necessary to measure the endogenous expression level of proteins in *C. albicans*. Experimental evidence of protein function and interactions under this condition is more reliable. C-terminal tagging is preferable as: 1) the possibility to affect normal protein folding is believed to be minimized (Huh et al., 2003); and 2) the possibility to affect the protein expression regulation is minimized (Ghaemmaghami et al., 2003). To study interactions among related proteins, it is often necessary to construct multiple-gene-tagged strains with two or more kinds of epitope tags. For this purpose, by using the *Exo* III-based ligase-free cloning method (Li and Evans, 1997), we introduced tandemly repeated 13Myc and 3HA tags into the upstream of *loxP*-flanked heterologous selection markers. A codon-optimized GFP was also introduced into the same position. A terminator element of the *ACT1* gene or the *ADH1* gene was placed downstream of the epitope tags. Ultimately, we obtained 9 vectors for C-terminal tagging (Fig. 1A).

To use these vectors, we adopted a modified PCR strategy to amplify the desired DNA cassettes. As shown in Fig. 1B, taking pCPC64 as an example, the F1 primer comprises two parts: 1) 20 bp homology to GFP; 2) 39 bp sequence precisely before the stop codon of the target gene. The R1, F2, and R2 primers could be easily designed as illustrated in Table S3. For the first round of PCR using F1/R1 primers, a product with 39 bp homology regions is generated. Using this product as PCR template directly, the second round of PCR using F2/R2 primers generates DNA cassettes with 78 bp homology regions to the target gene. This product could be transformed into *C. albicans* cells without further purification to generate a strain with the C-terminal tagged gene. Then, verification primers VP8 plus VP19 should be used for diagnostic PCR for pCPC64–66. VP6 plus VP19 should be used for pCPC61–63. VP7 plus VP19 should be used for pCPC58–60.

3.2. Doxycycline-inducible Tet-off promoter vectors

The Tet-on and Tet-off systems are two well-known regulatable promoters in mammalian cell research. They have been codon-optimized and adapted for *C. albicans* (Lai et al., 2011; Nakayama et al., 2000; Park and Morschhäuser, 2005). To facilitate our study of essential genes, we expanded the Tet-off promoter system. As shown in Fig. 2A, the TetR element was codon-optimized by chemical synthesis and fused to *GAL4AD* to generate catTA, which was then placed downstream of the *ADH1* promoter and upstream of the *ACT1* terminator. Conventional selection markers *URA3, HIS1* and *ARG4* were placed upstream