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Yeast Functional Analysis Report

Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in *Candida albicans*

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Abstract

We have developed a set of plasmids containing fluorescent protein cassettes for use in PCR-mediated gene tagging in *Candida albicans*. We engineered *YFP* and *CFP* variants of the *GFP* sequence optimized for *C. albicans* codon usage. The fluorescent protein sequences, linked to *C. albicans* auxotrophic marker sequences, were amplified by PCR and transformed directly into yeast. Gene-specific sequence was incorporated into the PCR primers, such that the tag-cassette integrates by homologous recombination at the 3′-end of the gene of interest. This technique was used to tag Cdc3 and Tub1 with GFP, YFP and CFP, which were readily visualized by fluorescence microscopy and localized as expected. In addition, Tub1–YFP and Cdc3–CFP were visualized in the same cells. Thus, this technique directs one-step construction of multiple fluorescent protein fusions, facilitating the study of protein co-expression and co-localization in *C. albicans* cells *in vivo*.
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Keywords: ***Candida albicans*; cyan fluorescent protein; epitope tagging; green fluorescent protein; polymerase chain reaction; protein co-localization; yellow fluorescent protein**

Introduction

Candida albicans is an important fungal pathogen of immunocompromised patients, causing superficial as well as invasive systemic disease. Virulence factors include adhesion to epithelial and endothelial cells, production of extracellular proteinases, and the ability to switch between yeast-form and filamentous morphologies (Odds, 1994). The study of the molecular mechanisms of these processes has been hindered by the fact that *C. albicans* reads the CUG codon as serine rather than leucine, rendering heterologous gene expression largely unsuccessful (De Backer *et al.*, 2000).

Green fluorescent protein (GFP) is widely recognized as a powerful tool in cell biology and serves as an important reporter for monitoring localization and expression of proteins in many organisms (Chalfie *et al.*, 1994; Niedenthal *et al.*, 1996; Valdivia *et al.*, 1996). The GFP variants yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) were engineered to improve brightness, provide additional wavelengths of emission, and facilitate multicolour imaging of differential gene expression and protein localization (Ellenberg *et al.*, 1998; Heim *et al.*, 1994; Heim and Tsien, 1996).

GFP sequences engineered with codons optimized for expression in *C. albicans* have been expressed either from a plasmid or from an integrated chromosomal locus (Cormack *et al.*, 1997; Morschhauser *et al.*, 1998). Both of these constructs employed conventional cloning techniques to generate a promoter–GFP fusion that could be transformed into *C. albicans*. In *C. albicans*, plasmids are highly unstable, variable in copy number from cell to cell, and rapidly lost in the absence of selective pressure (Cannon *et al.*, 1992; Kurtz *et al.*, 1987; Pla *et al.*, 1995). Thus, integrated single copy reporters are desirable. For example, a chromosomally integrated *GFP* construct expressed

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from the *SAP2* promoter was expressed only under conditions that promote Sap2 proteinase expression (Morschhauser *et al.*, 1998).

In *C. albicans*, gene deletion by homologous recombination is now a standard technique (Fonzi and Irwin, 1993). Recently, PCR-mediated strategies that allow rapid, efficient gene disruption or deletion have been developed (Wilson *et al.*, 2000, 1999). These methods use PCR primers with 5′-ends that correspond to the desired target gene sequences, and 3′-ends that direct amplification of the selectable marker gene. The amplified DNA is transformed directly into *C. albicans*, and recombinants that carry the inserted marker at the locus of interest are identified.

In this work, we combined PCR-mediated gene insertion, as developed for *C. albicans*, along with site-directed mutagenesis of *GFP* to construct codon-optimized *GFP* variants. We generated a set of plasmid cassettes that direct one-step construction of fluorescent protein fusions in *C. albicans*. We also demonstrate the use of these cassettes to generate strains expressing two distinguishable proteins in the same *C. albicans* cells.

Materials and methods

Strains, growth conditions and DNA methods

All strains were derived from BWP17 (*ura3*::*imm434*/*ura3*::*imm434 his1*::*hisG*/*his1*::*hisG arg4*::hisG/*arg4*::*hisG*) (Wilson *et al.*, 1999) and were grown at 30°C in rich (YEPD) medium, synthetic complete medium, or synthetic complete medium lacking specific nutrients (Sherman, 1991). *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA) and standard media and methods (Ausubel *et al.*, 1995) were used for plasmid manipulations. Yeast genomic DNA was isolated according to the method of Hoffman and Winston (1987). Polyacrylamide gel electrophoresis-purified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

DNA for plasmid constructions and yeast transformation was generated by PCR using the Qiagen Taq DNA polymerase kit (Qiagen). The 50 μl reaction mix contained 3.5 mM $MgCl_2$, 5 μl 10× kit buffer, 0.4 mM each dNTP, 10 μg BSA, 0.6 μM each primer, 2.5 U Taq DNA polymerase, and 0.1 μg plasmid template. Reactions were run for 1 cycle of 4 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min/kb of the desired product at 72°C.

Transformants were screened by PCR as above, except using 2 μM each of primer and 1 μl (~0.5 μM) of yeast genomic DNA.

Construction of *GFP* variants YFP and CFP

pMG871, containing the *C. albicans* codon-optimized *GFP* sequence in pYEGFP3 (Cormack *et al.*, 1997) ligated to *Hin*dIII/*Bam*HI-digested pUC119, was used to produce the template for site-directed mutagenesis (Kunkel, 1985; Kunkel *et al.*, 1987). The oligonucleotide primers used to generate pMG1416, which encodes YFP, were 5′-TCTAGCAAAACATTGTAAACCATAACCGAAAGT-3′ (V68L) and 5′-GGATAAGGCAGATTGGTAGGATAAGTAATGGTTGTC-3′ (T203Y). The oligonucleotides used to generate pMG1683, which encodes CFP, were 5′-AACACCCCAACCCAAAGTAGTGACTAA-3′ (F64L), 5′-ACATTGAACACCCCAACCGAAAGTAGT-3′ (Y66W), 5′-CATTGTGAGAAATATAGTTGTATTCC-3′ (N146I), 5′-GTTTGTCAGCAGTGATGTAAACATTG-3′ (M153T), 5′-CTAATTTTGAAGTTAGCTTTGATACCATTC-3′ (V163A) and 5′-TCTAATTTTGAAGTGAGCTTTGATACC-3′ (N164H).

Plasmid constructions

To generate pMG1506, which includes *GFP* and *URA3* sequences, the *Bam*HI–*Pvu*I fragment of plasmid pMG871 was ligated to the *Bcl*I–*Pvu*I *URA3* fragment of pVEC (gift of P. and B. Magee, University of MN). To introduce the *C. albicans ADH1* terminator after *GFP*, the *ADH1* terminator in pYPB1–ADHp1 (Bertram *et al.*, 1996) was isolated as a *Pst*I–*Eco*RI fragment and ligated to *Pst*I–*Eco*RI-cut pMG1506, between the *GFP* and *URA3* sequences, to generate pGFP–URA3 (Figure 1).

To generate pGFP–HIS1, the *C. albicans HIS1* sequence in pGEM–HIS1 (Wilson *et al.*, 1999) was amplified by PCR using the following primers, which were engineered to contain *Eco*RI sites (shown in **bold**): forward, 5′-GGACCG**GAATTC**CGGGGATCCTGGAGGATGAGGAGA-3′; reverse, 5′-GGACCG**GAATTC**CGGAATATTTATGAGAAACTATCA-3′ The resulting PCR product was digested with *Eco*RI and ligated to *Eco*RI-digested pGFP–URA3 to generate pGFP–HIS1 (Figure 1).

Using analogous approaches, the *YFP* and *CFP* sequences contained in pMG1416 and pMG1683, respectively, were used to generate pYFP–URA3,

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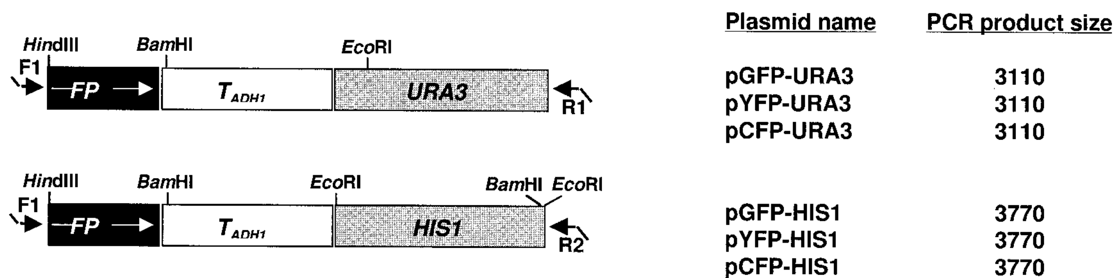

Figure 1. PCR template cassettes used to generate tags at the 3′-ends of *C. albicans* genes. Black boxes, codon-optimized *GFP*, *CFP* or *YFP*; white boxes, *C. albicans ADH1* termination sequence (T_{ADH1}); grey boxes, *C. albicans* auxotrophic markers (*URA3*, *HIS1*). Oligonucleotide primers (F1, R1, R2) used to generate the tag cassettes by PCR are described in Materials and methods and Table 1

pYFP–HIS1, pCFP–URA3 and pCFP–HIS1 (Figure 1).

Transformation of *C. albicans* and identification of integration events

PCR was performed using one of the cassettes shown in Figure 1 as the template, and the appropriate target-gene-specific primer pairs were designed as indicated in Figure 1 and Table 1. The products from 10 PCR reactions were pooled, extracted once with chloroform, precipitated with ethanol, resuspended in 20 μl of water and used to transform *C. albicans* strain BWP17 (Wilson *et al.*, 1999). Transformants were selected by plating the transformation mix on the appropriate selective medium. To identify transformants in which the cassette had correctly integrated into the target gene sequence, genomic DNA was prepared and used as the template in PCR reactions, using one primer that annealed within the transformation module and a second primer that annealed to the target gene locus outside the altered region.

Gene-specific sequences used to generate tag cassettes for *CDC3* and *TUB1*

To tag *CDC3* with *GFP*, *YFP* and *CFP*, *CDC3*-specific sequence was added to the universal primer sequences as described in Table 1. To tag *TUB1* with *GFP*, *YFP*, and *CFP*, *TUB1*-specific sequence was added to the universal primer sequences as described in Table 1.

Morphological observations

Differential interference contrast microscopy and epifluorescence microscopy were performed using a

Table 1. PCR primers used to amplify the transformation cassettes

Primer	Primer sequence
F1	5′-(gene-specific sequence) **GGT GGT GGT** TCT AAA GGT GAA GAA TTA TT-3′[a]
R1	5′-(gene-specific sequence) TCTAGAAGGACCACCTTTGATTG-3′
R2	5′-(gene-specific sequence) GAATTCCGGAATATTTATGAGAAAC-3′
CDC3-forward	5′-ACAAAAATTATTACCACAAGACCCACCAGCACAACCAGCTCCACAAAAGAGT CGTAAAGGATTTTTACGT-3′+F1
CDC3-reverse	5′-AATTAAACAAACAGATTAACAAACAAATAAACTAAATTAAGTTACATACTATTT AGCTATACCTCGGCCC-3′+R1 or R2
TUB1-forward	5′-CTTGGCTGCTTTAGAGAGAGATTATATTGAAGTTGGTACTGATTCTTTCCCTGA AGAAGAAGAAGAATAT-3′+F1
TUB1-reverse	5′-ATAATGAAAAACCCAGACCTTTGTAATTAAAAAAATTTAAACATTAGCAACAAA GTAAGAACACGATCAA-3′+R1 or R2

Primer combinations, orientations and locations relative to the plasmid templates are indicated in Figure 1. The gene-specific sequences included in the primers used in this study were approximately 70 nucleotides in length. To tag full-length proteins, the gene-specific sequences of the forward primer end just upstream of the stop codon, preserving the reading frame of the tag, whereas those of the reverse primer are chosen to end just downstream of the stop codon.

[a]The reading frame is indicated by spacing within the sequence; the sequence encoding the glycine linker is shown in **bold**.

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Nikon Eclipse E800 photomicroscope equipped with a 100 W mercury lamp, and epifluorescence illumination with green fluorescent protein (GFP) (excitation filter 470–490 nm, barrier 520–580 nm), blue fluorescent protein/cyan fluorescent protein (CFP) (excitation filter 380–400 nm, barrier 435–485 nm) and yellow fluorescent protein (YFP) (excitation filter 490–510 nm, barrier 520–550 nm) filter sets (Chroma Technology Corp., Brattleboro, VT). Digital images were collected using a CoolCam liquid-cooled, three-chip colour CCD camera (Cool Camera Company, Decatur, GA) and captured to a Pentium II 300 MHz computer, using Image Pro Plus version 4.1 software (Media Cybernetics, Silver Springs, MD). Images were processed using Adobe Photoshop version 5.5 (Adobe Systems Corp., San Jose, CA).

Results and discussion

PCR template plasmids for gene tagging

Cormack *et al.* (1997) developed a *GFP* sequence in which the 239 codons of the *Aequorea victoria GFP* gene (Chalfie *et al.*, 1994) were optimized for expression in *C. albicans*. In addition to the codon optimization, two additional mutations were made (S65G and S72A) to enhance the brightness of the expressed protein. We modified this codon-optimized *GFP* sequence using the mutations necessary to effect colour changes, from green to yellow or from green to cyan, that were developed and described for GFP variants used in mammalian expression systems (Ellenberg *et al.*, 1998; Heim *et al.*, 1994; Heim and Tsien, 1996). Thus, we introduced the same mutations, by site-directed mutagenesis of the codon-optimized GFP sequence, to generate the *C. albicans* codon-optimized versions of *YFP* and *CFP*.

To facilitate the use of these sequences in tagging multiple genes differentially at the carboxy-terminus of their native genomic loci, we developed a set of PCR template cassettes, each containing one of the fluorescent proteins and one of two selectable markers, *URA3* or *HIS1* (Figure 1). Thus, by using a *C. albicans* strain with auxotrophies for *ura3* and *his1*, two genes can be tagged within the same strain.

Validation of the system

We wanted to construct GFP, YFP and CFP fusion proteins to study gene expression and protein localization in *C. albicans*. To show that these fluorescent proteins are efficiently expressed and visualized in *C. albicans*, we used the cassettes described in Figure 1 to tag *C. albicans CDC3* and *TUB1*. *CDC3* encodes the homologue of the *S. cerevisiae* septin Cdc3, a protein that localizes to the mother-bud neck (Field and Kellogg, 1999; Longtine *et al.*, 1996). *TUB1* encodes the homologue of *S. cerevisiae* tubulin, a cytoskeletal protein that localizes to the mitotic spindle as well as to cytoplasmic microtubules (Schatz *et al.*, 1988).

Cdc3–GFP was generated using the pGFP–URA3 cassette and the *CDC3* forward and reverse primers listed in Table 1. *C. albicans* strain BWP17 was transformed with the PCR product and Ura^+ transformants were verified by PCR. Fluorescence microscopy revealed that Cdc3–GFP localized at the mother-bud necks of yeast cells (Figure 2A) in a cell cycle-dependent manner, similar to the localization pattern reported for *S. cerevisiae* Cdc3. Of note, Cdc3–GFP was observed in 100% of cells with small or medium-sized buds. Similarly, when pYFP–URA3 or pCFP–URA3 were used as templates with *CDC3* primers, we observed yellow or cyan fluorescent signal at the mother-bud neck of cells (Figure 2A). In addition, an abundant non-cell cycle-regulated protein was expressed and visualized in $>96\%$ of cells (data not shown).

We used GFP, YFP and CFP cassettes to also tag *TUB1* in *C. albicans*, and observed the characteristic localization of mitotic spindles for all three tubulin-fluorescent protein fusions (Figure 2B and data not shown). Of note, the efficiency of homologous integration into the gene of interest, determined by PCR screening, was approximately 30–40% of the original transformants. Thus, the codon-optimized GFP, as previously shown by Cormack *et al.* (1997), as well as the codon-optimized YFP and CFP, are efficiently expressed and visualized in *C. albicans* when expressed within the genomic context of a particular gene.

To determine whether YFP and CFP fusion proteins could be localized simultaneously within the same cell, we generated a strain carrying both *CDC3–CFP* and *TUB1–YFP* by sequential transformation. Fluorescence microscopy using cyan- and yellow-specific filter sets detected a cyan signal at the mother-bud neck corresponding to Cdc3, and either a punctate or a linear yellow signal within the cell cytoplasm corresponding to the mitotic spindle prior to or during anaphase, respectively

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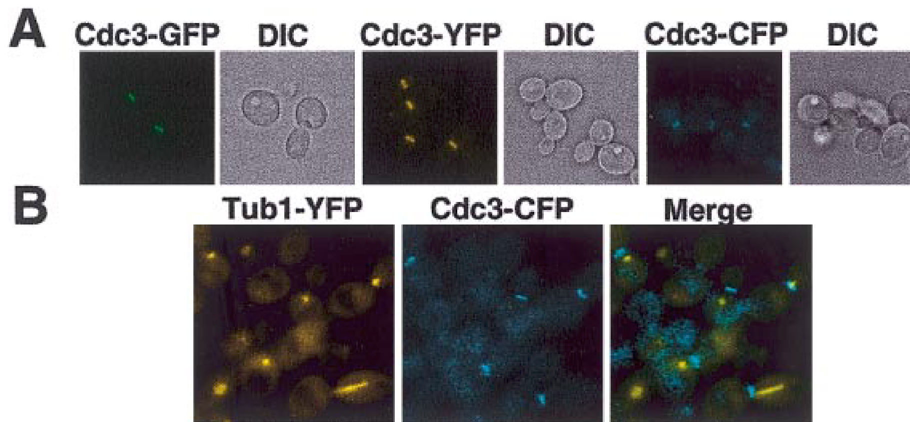

Figure 2. Analysis of fluorescent protein fusions in *C. albicans*. (A) Cdc3 tagged with GFP (left), YFP (centre) and CFP (right). The corresponding differential interference contrast (DIC) micrographs are shown to the right of each fluorescence micrograph. (B) Tub1 tagged with YFP (left), Cdc3 tagged with CFP (centre) and the merged image (right)

(Figure 2B). Thus, our codon-optimized YFP and CFP can be used simultaneously within the same cell to assess the expression of two proteins and/or the degree of co-localization of two proteins.

Conclusions

We have described a set of plasmids containing fluorescent protein cassettes that are useful as templates for PCR-mediated gene tagging in *C. albicans*. Use of these cassettes results in the generation of a protein tagged at the carboxy-terminus that can be visualized *in vivo* in *C. albicans* cells. Furthermore, the GFP epitope may provide a more efficient way to isolate and visualize proteins in *C. albicans* cell lysates using commercially available anti-GFP antibodies.

The codon-optimized GFP variants YFP and CFP are important tools that will facilitate the study of protein co-localization and co-expression in *C. albicans*. In addition, similar to what has been found for gene disruption strategies (Wilson *et al.*, 2000, 1999), we found that PCR-mediated gene tagging is a faster and more efficient way to generate fusion proteins in *C. albicans* than using conventional cloning techniques. Our method of tagging genes at the native genomic locus offers the opportunity to do time-lapse studies of protein localization *in vivo*, and to analyse the expression of proteins from their native promoters. The ability to do time-lapse studies of protein localization and expression in *C. albicans* is a significant technological advance that will aid our efforts to understand the biological changes that are required for the process of morphogenesis.

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