

## NOTE

# A Photostable Green Fluorescent Protein Variant for Analysis of Protein Localization in *Candida albicans*<sup>∇</sup>

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Received 4 November 2009/Accepted 8 November 2009

**Fusions to the green fluorescent protein (GFP) are an effective way to monitor protein localization. However, altered codon usage in *Candida* species has delayed implementation of new variants. Examination of three new GFP variants in *Candida albicans* showed that one has higher signal intensity and increased resistance to photobleaching.**

The human fungal pathogen *Candida albicans* can cause severe infections, particularly in immunocompromised patients. Important insights into its pathogenesis have been obtained by analyzing fusions to green fluorescent protein (GFP) (8). Although GFP tagging has been very successful, many fusion proteins are not easily detected. New GFP variants with improved fluorescence and protein folding properties have been identified by genetic approaches in other organisms (2, 7, 8). However, these GFP variants have not been assessed in *C. albicans* and related species, presumably because of the added difficulties of attempting heterologous expression in *C. albicans*.

To adapt GFP for effective use in *C. albicans*, Cormack et al. introduced three types of codon changes: the S65G S72A mutations to enhance fluorescence; the CTG codon 201 change to TTG, since CUG is translated as Ser instead of Leu in *C. albicans*; and the optimization of the other codons for translation in *C. albicans* (1). This variant, known as YeGFP3, was introduced into convenient vectors for creating gene fusions in *C. albicans* (4). Another version of eGFP known as mut2 (S65A V68L S72A Q80R) was adapted for *C. albicans* by changing the CTG codon but without further codon optimization (5). These obstacles to heterologous expression in *C. albicans* have presumably delayed implementation of newer versions of GFP. Therefore, in this study three different GFP variants were introduced into YeGFP3 and examined for function in *C. albicans*.

The GFP variants were constructed using standard methods to introduce changes in the coding sequence of YeGFP3. In brief, mutagenic oligonucleotides were used to prime PCR synthesis of a plasmid carrying YeGFP3, the template DNA was then destroyed by digestion with DpnI, and then the resulting DNA was transformed into *Escherichia coli*. DNA sequencing (carried out by the Stony Brook University DNA

Sequencing Facility) confirmed that the correct substitutions were present. The mutant GFP genes were then released as PstI-AscI fragments and then were subcloned to replace the corresponding GFP fragment of plasmid pFa-GFP-URA3 (6), which carries a PCR cassette module for creating GFP fusions in *C. albicans*. Because of the large number of changes, the mutants were given the more convenient names of CaGFP $\alpha$  (F64L S65T F99S M153T V163A), CaGFP $\beta$  (F64L S65T N149K M153T I167T; also known as emerald), and CaGFP $\gamma$  (F64L S65C V163A I167T). The CaGFP $\gamma$  was also introduced into vectors that contain selectable markers *HIS1* and *ARG4* (6). DNA sequences used to design primers for creating GFP fusions in *C. albicans* were as follows: forward primer, 5' (region of homology)-GGTGCTGGCGCAGGTGCTTC-3', and reverse primer, 5' (region of homology)-TCTGATATCATCG ATGAATTCGAG-3'.

*CDC11-GFP* fusion genes were created in *C. albicans* by homologous recombination, as described previously (4, 6). In brief, long oligonucleotide primers with homology to the 3' end of the *CDC11* open reading frame were used to prime PCR synthesis of each of the corresponding GFP variant genes plus an adjacent selectable marker gene (*URA3*). These DNA elements were then introduced into *C. albicans* cells and allowed to recombine with the homologous region of the *CDC11* gene in *C. albicans* to create the *CDC11-GFP* fusion genes. Sequences used for the design of PCR primers to amplify the pFa-GFP plasmids are shown above. Cells carrying the indicated *CDC11-GFP* fusion gene were grown overnight in log phase in synthetic medium (yeast nitrogen base plus amino acids and dextrose). Cdc11-GFP fluorescence intensity was analyzed with an Olympus BH2 microscope equipped with a Zeiss AxioCam camera run by Openlab software. The relative GFP signal was determined by measuring the intensity of GFP fluorescence of the septin ring and then subtracting the fluorescence of an area immediately adjacent to each ring. All samples were visualized under the same conditions.

Samples were prepared for Western blot analysis by resuspending cells in TNE lysis buffer (10 mM Tris base, 1 mM

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<sup>∇</sup> Published ahead of print on 13 November 2009.

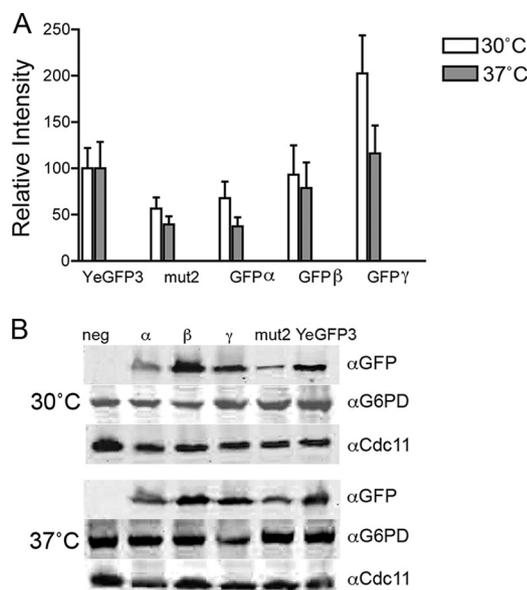


FIG. 1. Properties of Cdc11-GFP fusion proteins. Cells were grown to log phase overnight at the indicated temperature, and then Cdc11-GFP fluorescence was analyzed. (A) Signal intensity for the different versions of Cdc11-GFP was compared in three independent assays in which 50 septin rings per assay were quantified for each different Cdc11-GFP. The average fluorescence intensity was normalized to 100 for Cdc11-YeGFP3. The Cdc11-CaGFP $\gamma$  variant gave a significantly stronger signal than the other variants ( $P < 0.001$ ). (B) Western blot analysis comparing the levels of Cdc11-GFP produced in the indicated strains. The lane labeled “neg” refers to the negative-control strain (BWP17) that lacks GFP. Blots were probed with anti-GFP to detect Cdc11-GFP, anti-glucose-6-phosphate dehydrogenase ( $\alpha$ G6PD) as a control, and anti-Cdc11 to detect the untagged version of Cdc11.

EDTA, 100 mM NaCl) with 100 $\times$  protease mix (40 mg/ml pepstatin A, 40 mg/ml aprotinin, 20 mg/ml leupeptin) and then agitating in the presence of glass beads. The supernatant was collected after low-speed centrifugation at 3,000 rpm for 1 min, protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce), and then equal amounts of protein extract were separated by gel electrophoresis and transferred to a Protran nitrocellulose membrane (Whatman GmbH). The blots were incubated with mouse anti-GFP (Millipore), rabbit anti-glucose-6-phosphate dehydrogenase (anti-G6PD; Sigma), or rabbit anti-Cdc11 (Santa Cruz Biotechnology) primary antibodies; washed; and then incubated with either goat anti-mouse IRDye 800cw or goat anti-rabbit IRDye 680 (Li-Cor Biosciences, Lincoln, NE). The immunoreactive proteins were visualized with a Li-Cor fluorescence scanner run by Odyssey software.

Three new GFP variants based on YeGFP3 were constructed by introducing mutations predicted to improve either the fluorescence properties or protein folding (2, 7, 8). Because multiple changes were introduced into each variant, they were given the more convenient names of CaGFP $\alpha$ , CaGFP $\beta$ , and CaGFP $\gamma$  (see above). The key mutations in CaGFP $\alpha$  and CaGFP $\beta$  have been described previously (2, 7, 8), but CaGFP $\gamma$  represents a novel combination of mutations. The 3 new GFP variants plus the YeGFP3 and mut2 versions were compared by fusing them to the C terminus of the Cdc11 septin protein (3). The Cdc11 protein was selected because its restricted

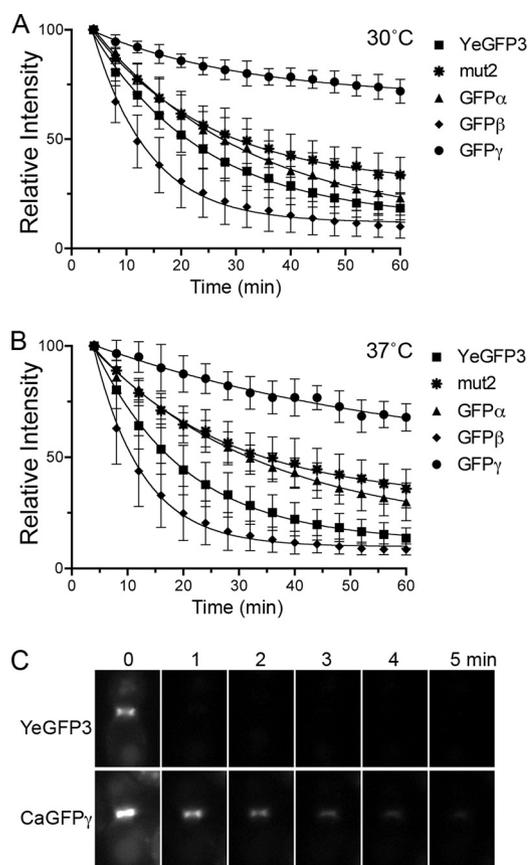


FIG. 2. Photostability of GFP variants. (A and B) Relative fluorescence intensity of the GFP variants at 4-s intervals over a time course of 1 min of continuous exposure to the fluorescence excitation lamp after growth at 30°C (A) and at 37°C (B). CaGFP $\gamma$  showed the best photostability ( $t_{1/2}$  of  $\sim 2$  min). The relative fluorescence was normalized to 100 for each Cdc11-GFP variant at the start of the time course. The results represent the average of three independent assays in which three septin rings were analyzed for each mutant. Error bars indicate standard deviations. (C) Cells carrying Cdc11 fused to YeGFP3 or CaGFP $\gamma$  were continuously exposed to the fluorescence excitation lamp, and then images of septin rings were captured at the indicated times.

localization to the bud neck facilitated microscopic analysis and comparison of fluorescence properties. *CDC11-GFP* fusion genes were constructed in strain BWP17 (9) using PCR-generated modules with a *URA3* selectable marker, as described previously (4, 6).

Cells were grown in synthetic medium overnight to log phase at both 30°C and 37°C, temperatures that are commonly used to propagate *C. albicans* and that may affect the folding properties of GFP. GFP fluorescence was then analyzed by quantifying the intensity of the septin rings in digital images (Fig. 1A). Septin rings were analyzed only if they were obviously in focus and at the same stage of the cell cycle (large budded). CaGFP $\gamma$  gave a slightly stronger signal than the other variants, which was most obvious at 30°C (Fig. 1A). At least two independent clones were analyzed for each *CDC11-GFP* variant, and the two gave similar results (data not shown).

The levels of the Cdc11-GFP proteins at both 30°C and 37°C were compared on two independent Western blots using

anti-GFP antibody (Fig. 1B). The relative levels of Cdc11-mut2GFP and Cdc11-CaGFP $\alpha$  were the lowest, consistent with their lower fluorescence intensity. The lower levels of Cdc11-mut2GFP are consistent with the fact that the codons in the mut2 version of GFP were not optimized for expression in *C. albicans* (5). The Cdc11-YeGFP3 and Cdc11-CaGFP $\gamma$  were present at higher levels, and the Cdc11-CaGFP $\beta$  was produced at even slightly higher levels, consistent with reports that this latter version of GFP (also known as emerald) has improved folding properties (7). The Cdc11-GFP variants did not affect the production of the untagged Cdc11 protein (Fig. 1B).

Photobleaching is also an important factor for GFP (7), especially in time-lapse studies or Z-stack analysis of different optical sections of cells. Photostability of the GFP variants was examined by taking pictures at 4-s intervals during 1 min of continuous exposure to the fluorescence excitation lamp (Fig. 2A and B). The fluorescence of YeGFP3, mut2GFP, and CaGFP $\alpha$  fused to Cdc11 decayed to 50% of original intensity within 15 to 30 s, and the rate of photobleaching was even higher for CaGFP $\beta$ . In contrast, Cdc11-CaGFP $\gamma$  showed extended photostability at both 30°C and 37°C (half-life [ $t_{1/2}$ ] of  $\sim 2$  min). Similar results were also obtained for CaGFP $\gamma$  fused to the Golgi protein Vrg4 (data not shown), although the standard deviations were larger because the mobile Golgi compartments frequently moved out of the focal plane during the time course (data not shown). On a practical level, the Cdc11-GFP $\gamma$  fluorescence was readily detectable after several minutes of continuous exposure (Fig. 2C), demonstrating its clear advantage for allowing more time to observe protein localization before photobleaching becomes significant.

Altogether, Cdc11-CaGFP $\gamma$  had the best overall properties based on protein levels, signal intensity, and photostability in *C. albicans*. The higher level of Cdc11-CaGFP $\beta$  production

was apparently offset by increased photobleaching, resulting in no overall advantage for this variant. The Cdc11-CaGFP $\alpha$  was produced at relatively low levels, and it was less photostable compared to the other versions. Thus, CaGFP $\gamma$  is a novel GFP variant that offers improved features for the study of protein localization in *C. albicans* and will likely also be useful for expression in other species.

We thank Jurgen Wendland, Cheryl Gale, and Judith Berman for plasmids and the members of our lab and Neta Dean for comments on the manuscript.

This work was supported by grant RO1 AI47837 from the National Institutes of Health that was awarded to J.B.K.

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