Fungal Genetics and Biology 92 (2016) 14–25

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Tools and techniques

Oxygen-independent FbFP: Fluorescent sentinel and oxygen sensor component in *Saccharomyces cerevisiae* and *Candida albicans*

Isabel Eichhof, Joachim F. Ernst *

Department Biologie, Molekulare Mykologie, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany

ARTICLE INFO

Article history:
Received 8 October 2015
Revised 22 April 2016
Accepted 23 April 2016
Available online 25 April 2016

Keywords:
FbFP
Hypoxia
Candida albicans
Saccharomyces cerevisiae
Nuclear localization
Surface display
Oxygen sensor

ABSTRACT

FMN-binding fluorescent proteins (FbFPs) outperform GFP and its derivatives because of their oxygen-independence, small size and rapid maturation. FbFPs have been used successfully as reliable reporters of gene expression in the cytoplasm of pro- and eukaryotes. Here we extend previous findings on the codon-adapted CaFbFP variant, which functions in the apathogenic yeast *Saccharomyces cerevisiae* and the human fungal pathogen *Candida albicans*. In both fungal species, CaFbFP could be targeted to the nucleus and the cell wall by endogenous signals (H2B-/Aga2-fusions) demonstrating its use as a fluorescent beacon in these relevant cellular locations. Transformants of both fungal species producing a CaFbFP-YFP fusion (YFOS) showed variable energy transfer from CaFbFP to YFP (FRET) that depended in its extent on external O_2 concentrations. Applications as fluorescent sentinel and oxygen biosensor expand the FbFP toolbox to study oxygen-independent cellular processes under hypoxia.

1. Introduction

Oxygen availability is known to regulate the metabolism and growth of eukaryotic cells. Both biotechnological applications and virulence traits of fungi are influenced by the presence or absence of molecular oxygen. Under hypoxia, yeast-type fungi switch to a fermentative mode of energy production and several metabolic pathways requiring molecular oxygen, e.g. those for the biosynthesis of unsaturated fatty acids and sterols, become limiting for growth (Ernst and Tielker, 2009; Grahl et al., 2012). The production of ethanol, isobutanol and various foods by the yeast *Saccharomyces cerevisiae* are examples for biotechnological processes requiring hypoxic fermentation conditions (Chen et al., 2011); furthermore, the efficiency of heterologous production of glucoamylase and α-amylase by this yeast was found to become improved under oxygen-limitation (Cha et al., 1997; Liu et al., 2013). In contrast to useful apathogenic yeast and filamentous fungi, the pathogen *Candida albicans* can harm humans by causing tenacious or even life-threatening disease (Wisplinghoff et al., 2014). This pathogen is a harmless and regular commensal mainly at low-oxygen concentrations in the gut (Kullberg and Oude Lashof, 2002; Scanlan and Marchesi, 2008; Ghannoum et al., 2010; Iliev et al., 2012) but in immunocompromised patients it can grow invasively and proliferate massively in other body niches and organs that vary in oxygen concentrations (Ernst and Tielker, 2009; Grahl et al., 2012). Recent results have revealed specific transcriptional circuits controlling gene expression under hypoxia, which determine the dimorphic growth and invasive potential of this pathogen (Setiadi et al., 2006; Desai et al., 2015). Further research on molecular processes occurring under low oxygen conditions in apathogenic and pathogenic fungi is required to improve both biotechnological applications and antifungal therapeutic treatments.

In recent years, a new class of oxygen-independent fluorescence reporters, flavin mononucleotide-binding fluorescence proteins (FbFPs), was developed on the basis of bacterial blue light receptors (Mukherjee and Schroeder, 2015; Buckley et al., 2015). These proteins carry a LOV (light oxygen voltage)-domain binding the cofactor FMN as chromophore, whose synthesis and fluorescence does not require oxygen and enables FbFPs to fluoresce under various oxygen conditions (Drepper et al., 2013). In contrast, the autocatalytic chromophore formation of GFP and its many derivatives is relatively slow and requires molecular oxygen (Heim and Tsien, 1996). To make use of the *Bacillus subtilis* blue light receptor YtvA, its effector domain was deleted and the photoactive cysteine of the LOV domain, which downregulates fluorescence by binding FMN in the photocycle, was exchanged to an alanine residue to enhance fluorescence (Drepper et al., 2007). The resulting protein EcFbFP (*Escherichia coli* FbFP) was found to be an effective and

* Corresponding author.
E-mail address: joachim.ernst@uni-duesseldorf.de (J.F. Ernst).

http://dx.doi.org/10.1016/j.fgb.2016.04.004
1087-1845/

fast-maturing, oxygen-independent reporter of gene expression in *E. coli*; recently, EcFbFP was also found to function in the cytoplasm of mammalian cells (Walter et al., 2012). In addition, the low mass of FbFPs (about 16 kDa) compared to GFP-variants (27 kDa) was found advantageous for some applications (Chapman et al., 2008). In the following, LOV-based proteins were developed further to generate high intracellular concentrations of reactive oxygen (Shu et al., 2011) or as sensors for metals and pH (Liu et al., 2014; Ravikumar et al., 2015). Importantly, Potzkei et al. (2012) found that a fusion of oxygen-independent EcFbFP to YFP acts as an oxygen sensor because of Förster resonance energy transfer (FRET) from EcFbFP to YFP occurs only in the presence of oxygen. We previously established the fluorescent protein CaFbFP (*C. albicans* FbFP) for oxygen-independent applications in *S. cerevisiae* and *C. albicans* (Tielker et al., 2009). For this purpose, the *EcFbFP* sequence was adapted by raising the AT-amount and by exchanging the seven CUG codons to UUG codons because in *C. albicans* CUG encodes serine instead of leucine (Santos et al., 1993). CaFbFP was found suitable to fluorescently label the cytoplasm of *S. cerevisiae* and *C. albicans* cells under both normoxic and anoxic conditions. In this study we further extend the potential application of CaFbFP as fungal reporter protein by demonstrating that not only the cytoplasm but also two additional cellular locations, i.e. the nucleus and the cell surface (cell wall), can be targeted by CaFbFP to monitor site-specific fluorescence in *S. cerevisiae* or *C. albicans*. Furthermore, we show that a CaFbFP-YFP fusion protein in the cytoplasm or on the cell surface acts as a live biosensor to monitor oxygen levels in- and outside of fungal cells.

2. Material and methods

2.1. Strains and growth conditions

Genotypes of *S. cerevisiae* strain EBY100 (Boder and Wittrup, 2000), *S. cerevisiae* THY.AP4 (Obrdlik et al., 2004) and *C. albicans* CAI4 (Fonzi and Irwin, 1993) are listed in Table S1. *S. cerevisiae* THY.AP4 cells were grown in SD medium (0.67% yeast nitrogen base, 2% glucose) or SGR (0.67% yeast nitrogen base, 2% galactose, 1% raffinose) supplemented with adenine and amino acids Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, Val but lacking uracil (SD ura$^-$; SGR ura$^-$) at 30 °C, whereas EBY100 was grown at 21 °C (30 °C under hypoxic conditions) on SD or SGR medium containing leucine (SD + Leu; SGR + Leu). 2% agar was added for plating. *C. albicans* CAI4 cells were grown in SD media at 30 °C. Growth of liquid cultures under hypoxic conditions (10, 4 or 0.2% oxygen) and 0.1% carbon dioxide was carried out in an INVIVO$_2$ 200 incubator (Ruskinn). In case of oxygen determination experiments, shake flasks were sealed with parafilm instead use of conventional lids to prevent oxygen exchange. Expression of *GAL1p* in *S. cerevisiae* THY.AP4 or EBY100 transformants was induced by inoculating cells of an overnight culture grown in SD ura$^-$ or SD + Leu medium in glucose-free, galactose-containing SGR ura$^-$ and SGR + Leu medium, respectively. Cells were grown to the exponential growth phase (OD$_{600}$ = 0.6–0.9) to ensure optimal protein synthesis, whereas under normoxic conditions *S. cerevisiae* EBY100 was grown for 23 h. *C. albicans* CAI4 cells were treated comparably to *S. cerevisiae* THY.AP4, inducing expression of *TDH3p* using glucose-containing SD medium for pre- and main cultures.

2.2. S. cerevisiae CaFbFP expression vectors

Plasmids are listed in Table S2 and oligonucleotides are listed in Table S3. *S. cerevisiae* expression vectors were constructed as follows: (i) *Cytoplasmic localization:* pIE3 carrying a single Ca*FbFP* ORF under control of *GAL1* promoter has been described (Tielker et al., 2009). For construction of expression vectors pIE3-2, pIE3-3 and pIE3-4 carrying two, three or four tandem copies of Ca*FbFP* the respective *CaFbFP* inserts were excised from plasmids pIE1-2 (Tielker et al., 2009), pGEM-T-3×CaFbFP or pIE5-4 using *Bgl*II and *Xho*I (pIE3-2, pIE3-3) or *Bgl*II and *Bam*HI (pIE3-4); the resulting fragments were cloned downstream of the *GAL1* promoter of the *S. cerevisiae* expression plasmid p426GAL1 (Mumberg et al., 1994). A threefold fusion of Ca*FbFP* was constructed by amplification of 2×Ca*FbFP* using Oligo CaFbFP-ABB-AvaI-weg-FW and a reverse primer (CaFbFP-ABB-AvaI-weg-RV) lacking the *Ava*I site upstream of the stop codon. The resulting fragment was cloned into pGEM-T (Promega GmbH). This plasmid was cut with *Ava*I between both Ca*FbFP* reading frames and a single Ca*FbFP* copy lacking the stop codon was inserted, which had been PCR-amplified using primers that add *Ava*I sites at the end of the fragment (CaFbFP-AvaI-FW/RV) resulting in pGEM-T-3×CaFbFP. (ii) *Nuclear localization:* pIE3-HTB1 and pIE3-2-HTB1 carrying a fusion of *HTB1* encoding histone H2B to either Ca*FbFP* or 2×Ca*FbFP* were constructed by first PCR-amplifying *HTB1* from gDNA of *S. cerevisiae* THY.AP4, using primers (ScHTB1-FW-neu/RV-neu) that generated fragments lacking the stop codon and flanking 65 bp-sequences at their 5′ and 3′ ends with homology to *GAL1p* and Ca*FbFP* sequences in plasmids pIE3 and pIE3-2, respectively. Subsequently, *HTB1* was inserted between *GAL1p* and Ca*FbFP* (2×Ca*FbFP*) by homologous recombination using co-transformation of the *HTB1* fragment with linearized (*Spe*I) pIE3 (pIE3-2) into *S. cerevisiae* strain THY.AP4. (iii) *Surface localization:* pIE9 carrying *AGA2*-Ca*FbFP* was constructed by amplification of Ca*FbFP* without stop codon but containing flanking *Eco*RI-*Xho*I sites using plasmid pGA18-CaFbFP as template (primers FFP-EcoRI-FW-opti/FFP-noStop-XhoI-RV-opt i). The fragment was subsequently cloned into the multiple cloning site downstream of *AGA2* and *Xpress* sequences in surface display vector pYD1 (Invitrogen). Multicopy plasmids pYD1-2μ, pIE9-2μ and pIE9K18A-2μ were constructed based on vectors pYD1, pIE9 and pIE9K18A by exchanging the *CEN6*/*ARS4* sequence against the *ori* sequence of the endogenous 2 μm plasmid. For this purpose, the 2 μm-*ori* sequence was PCR-amplified using plasmid YEp24 (Botstein et al., 1979) as template and primers 2mikron-vs-CEN6-FW/RV that generated 80 bp sequences at 5′ and 3′ fragment ends with homology to up- and downstream regions of the *CEN6*/*ARS4* sequence on the corresponding target vector. The *CEN6*/*ARS4* sequence of the target vectors was removed by digestion with enzymes *Ppu*MI and *Acc*I and the resulting fragments were co-transformed with the PCR fragment carrying the 2 μm-*ori* sequence into *S. cerevisiae* EBY100 to generate plasmids pYD1-2μ, pIE9-2μ and pIE9K18A-2μ by homologous recombination. Mutation of lysine18-encoding triplet AAA to alanine18-encoding GCA of Ca*FbFP* was done in plasmid pIE9 with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Canada) using primers CaFbFP-Lys-mutaPCR-FW/-RV. The resultant vector was named pIE9K18A. (IV) *YFOS:* Plasmids pOXY1, pOXY2, pOXY3 and pOXY4 were constructed by homologous recombination in *S. cerevisiae*. For this purpose, recipient vectors were linearized using enzymes *Spe*I (p426GAL1, pIE3), *Not*I (pYD1-2μ) or *Bam*HI (pIE9-2μ), respectively. For pOXY1 and pOXY2 a codon-optimized variant of *YFP* for the use in *C. albicans* (Gerami-Nejad et al., 2001) was amplified by PCR using primers (pOXY1: YFP-FW/YFP + linker-LP-RV; pOXY2: YFP-FW/YFP + linker-RV) generating 75 (5′)- or 63 bp (3′)-long sequences at the ends of the fragments with homology to *GAL1p* and sequences downstream of *GAL1p* in the plasmid or Ca*FbFP* sequences in the according target plasmid p426GAL1 and pIE3; plasmid pYFP-URA was used as template DNA. Additionally, these primers added a 42 bp linker sequence to the 3′ ends of the fragments (YFOS GenBank accession KU245541). To construct pOXY3 and pOXY4, *YFP* fused to the linker sequence was amplified using pOXY2 as template DNA and primers YFP + linker-pYD-FW/