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Tools and techniques

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

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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

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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/

RV (pOXY3) or primers YFP + linker-pYD-FW/YFP + linker-pIE9-RV (pOXY4), whereby 81 (5′) and 65 bp (3′) flanking sequences homologous to a region downstream of *AGA2* (including sequences of Xpress epitope) and Ca*FbFP* (pOXY4) or a sequence further downstream of *AGA2* (including sequence of V5 epitope) (pOXY4) were added. Linearized recipient vectors and PCR fragments were co-transformed into *S. cerevisiae* EBY100 to obtain the desired plasmids by homologous recombination (AGA2-YFOS GenBank accession KU255435).

2.3. C. albicans CaFbFP expression vectors

(i) *Cytoplasmic localization*, plasmids pIE5, pIE5-1 and pIE5-2 have been described (Tielker et al., 2009). Plasmid pIE5-3 was constructed analogously to pIE3-3, by inserting 3×Ca*FbFP* downstream of *TDH3* promoter into *Bgl*II site of *C. albicans* expression plasmid pIE5 (Tielker et al., 2009). Construction of pIE5-4 (4×Ca*FbFP*) was done similarly by insertion of a 2×Ca*FbFP* tandem gene into pIE5-2, resulting in a fusion of four Ca*FbFP* reading frames. (ii) *Nuclear localization: HTB1* was amplified from gDNA of *C. albicans* strain CAI4 using primers (CaHTB1-FW-BglII/-RV-BamHI) that added *Bgl*II-*Bam*HI sites to the fragment ends. Subsequent insertion of *HTB1* between *TDH3p* and 2×Ca*FbFP* or 4×Ca*FbFP* of plasmid pIE5-2 or pIE5-4 led to construction of pIE5-2-HTB1 and pIE5-4-HTB1. (iii) *YFOS variants:* For the construction of pOXY7, pOXY8, pOXY9 and pOXY11 *YFP* plus linker sequence were amplified by PCR using pOXY2 as template DNA and primers YFP + linker-FW-BglII/-RV-BamHI. The resulting fragment was inserted downstream of *TDH3p* in pIE5 (pOXY7-pre) or between *TDH3p* and Ca*FbFP* in pIE5-1 (pOXY8), 2×Ca*FbFP* in pIE5-2 (pOXY9) or 4×Ca*FbFP* in pIE5-4 (pOXY11). Additionally, in pOXY7-pre a translational stop codon for *YFP* was added by mutagenesis PCR using primers YFP + linker-Stopp-muta-FW/-RV generating pOXY7.

2.4. Protein methods

Crude extracts of cells in the exponential growth phase were obtained by harvesting cells by centrifugation (5 min; 3500 rpm) and resuspension in lysis buffer (25 mM Tris–HCl pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.1% Triton X-100; 10% glycerol) containing protease inhibitors (Complete Mini, Roche). Cells were broken by shaking with glass beads in a FastPrep homogenizer (MP Biochemicals) using 6 cycles of 40 s 6.5 m s^{-1} cooling samples on ice for 3 min after each cycle.

For immunodetection proteins in the crude extracts were separated by SDS-PAGE using 10% or 4–20% acrylamide gels (Pierce) and transferred to a PVDF membrane. Detection of CaFbFP or YFP was achieved using anti-EcFbFP antiserum (1:20,000) or a monoclonal anti-GFP antibody (1:1000), both derived from rabbit, followed by treatment with horseradish peroxidase-labeled secondary antibody (1:10,000) from goat (directed against rabbit). Light signals were detected with an ImageQuant LAS 400 mini imager (GE Healthcare).

2.5. Fluorescence analyses

Fluorescence measurements of crude extracts (400 μg protein) or whole cells (OD_{600} = 0.2 = 4 × 10^6 cells) were done in a volume of 200 μl in black 96-well microtiter plates using a Fluoroskan Ascent FL (Thermo Scientific) fluorometer with filters for excitation at 450 nm and fluorescence detection at 495 nm, according to the maxima of CaFbFP (Tielker et al., 2009). An Infinite M200 (Tecan Group Ltd.) fluorometer was used for fluorescence emission spectra (λ_{exc} = 380 or 430) as well as for control measurements of CaFbFP- (λ_{exc} = 450, λ_{em} = 495) and YFP-mediated (λ_{exc} = 500, λ_{em} = 530) fluorescence within these measurements. Furthermore, in case of emission spectra taken from whole cells, cells were washed with PBS (140 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 1.8 mM KH_2PO_4 (pH 7.4)) before measuring to avoid an influence of riboflavin, an ingredient of the minimal media. To record emission spectra of cells grown under hypoxic conditions or for oxygen determination experiments, washing steps using preincubated PBS, as well as filling of the 96-well plates, were done under hypoxic conditions and probes were instantly measured.

For fluorescence microscopic analyses yeast cells were grown for 1 d at 30 °C on SD (*C. albicans* CAI4), SGR ura$^-$ (*S. cerevisiae* THY.AP4) or SGR + Leu (*S. cerevisiae* EBY100) plates. A small amount of cells was dissolved in ProLong Gold antifade reagent (Invitrogen) and analyzed on glass slides using an Axio Imager. M1 or Axioscop 40FL fluorescence microscope (Zeiss) and Zeiss filter sets FS38HE for CaFbFP, FS46 for YFP or FS02 for DAPI staining. Some photographs of *C. albicans* cells with fluorescence-labeled nuclei were performed with a spinning disc microscope (Cell Observer SD, Zeiss) during excitation at 488 (CaFbFP) or 353 nm (DAPI) by an argon laser and detection of emission at 509 and 465 nm, respectively. Staining of cell nuclei was achieved by the addition of 1 μl DAPI (1 μg/ml) to 5 μl cell suspension followed by 10 min of incubation in the dark.

3. Results

3.1. Activity of tandem CaFbFP fusions in S. cerevisiae and C. albicans

Previously, a tandem fusion consisting of two CaFbFP sequences (2×CaFbFP) had shown stronger fluorescence than the single CaFbFP protein in *C. albicans* (Tielker et al., 2009). To further examine the effects of tandem repeats on fluorescence intensity we constructed expression vectors carrying multiple tandem Ca*FbFP* reading frames for *S. cerevisiae* and *C. albicans*. In *S. cerevisiae*, the inducible *GAL1* promoter was used to drive transcription of the respective single and tandem fusion genes; plasmids pIE3, pIE3-2, pIE3-3 and pIE3-4 contained one, two, three and four copies of Ca*FbFP*, respectively. Expression vectors were transformed into *S. cerevisiae* strain THY.AP4 and transformants were grown in *GAL1p*-inducing SGR Ura$^-$ minimal medium to the exponential growth phase; a transformant carrying empty vector p426GAL1 was treated similarly and served as negative control strain. Equal amounts of cell extracts (100 μg protein) were separated by SDS-PAGE (4–20% polyacrylamide) followed by immunoblotting using anti-EcFbFP antiserum (Fig. 1a, i). As expected from previous findings (Tielker et al., 2009), single and tandem CaFbFP migrated as bands of about 16 kDa and 36 kDa, close to the theoretical masses of 15.7 and 31.4 kDa, respectively (Fig. 1a, i). Furthermore, the synthesis of 3×CaFbFP and 4×CaFbFP (theoretical masses of 47.1 and 62.8 kDa) was shown by bands of about 55 kDa and 70 kDa, respectively. The CaFbFP signal intensity increased from one to three CaFbFP repeats in the fusion, which was probably caused in parts by increased antibody binding sites; unexpectedly, however, amounts of the 4×CaFbFP protein were lower than those of 2×CaFbFP and 3×CaFbFP proteins suggesting that CaFbFP protein levels in this fusion were affected differently by unknown factors. Fluorescence activity of CaFbFP fusions was assessed in crude extracts (400 μg protein) of six transformants, which were each measured in triplicate with filters for excitation at 450 nm and detection of emission at 495 nm (Drepper et al., 2007). Differences among transformants may be due to variable plasmid copy numbers (Schneider and Guarente, 1991). Comparisons of average fluorescence levels (lines in Fig. 1a, ii) indicate enhanced

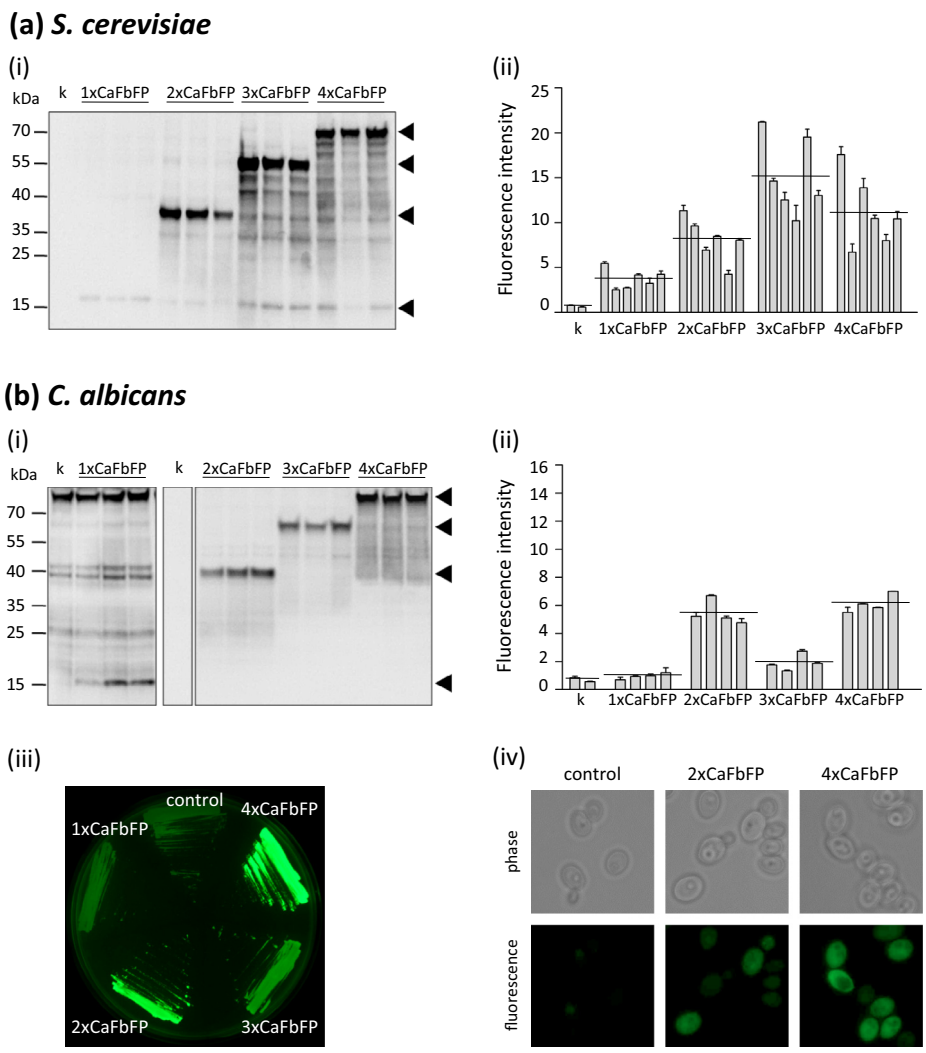

Fig. 1. Cytoplasmic production of FbFP fusions. (a) Host *S. cerevisiae*. Strain *S. cerevisiae* THY.AP4 carrying plasmid pIE3 (1×Ca*FbFP*), pIE3-2 (2×Ca*FbFP*), pIE3-3 (3×Ca*FbFP*) or pIE3-4 (4×Ca*FbFP*) were tested, in which *FbFP* genes are under control of the inducible Sc*GAL1* promoter; strain THY.AP4 carrying empty plasmid p426GAL1 was used as control strain (k). (b) Host *C. albicans*. Strain *C. albicans* CAI4 carrying either plasmid pIE5-1 (1×Ca*FbFP*), pIE5-2 (2×Ca*FbFP*), pIE5-3 (3×Ca*FbFP*) or pIE5-4 (4×Ca*FbFP*) were tested, in which *CaFbFP* genes are under control of the Ca*TDH3* promoter; strain CAI4 carrying empty plasmid pIE5 was used as control strain. (i) The presence of CaFbFP in crude cell extracts (100 μg protein) of several transformants was examined by SDS-PAGE followed by immunoblotting using anti-EcFbFP antiserum. Note that for strain CAI4 [pIE5-1] a threefold amount of crude extract (300 μg) was used and exposure time was prolonged to allow FbFP detection. Arrows indicate the migration of CaFbFP protein. (ii) Fluorescence measurements (λ_{exc} = 450 nm, λ_{em} = 495 nm) of crude extracts (400 μg protein) of several transformants; lines indicate average fluorescence intensities. (iii) *C. albicans* transformants producing the indicated CaFbFP variants were streaked out on SD agar and grown for 1 d at 30 °C before photography of the plate under excitation at 460 nm (ImageQuant LAS 4000). (iv) Phase contrast photographs of transformant cells under illumination with white light or fluorescence of cells during excitation with blue light (Zeiss filter set FS38HE, excitation: BP470/40, emission: 525/50).

fluorescence units (FU) for 2×CaFbFP (8.12 FU) compared to 1×CaFbFP (3.74 FU) and a further increase for 3×CaFbFP (15.19 FU). As expected from immunoblotting, fluorescence of 4×CaFbFP (11.18 FU) was not greater than the 2× and 3× variants lead; which may reflect lower CaFbFP protein levels or an altered three dimensional conformation of FbFP that may hinder attachment of FMN to the binding pocket.

Using *C. albicans* as the production host, the previously described strains CAI4[pIE5-1] and CAI4[pIE5-2] were used that express either single or tandem Ca*FbFP* under control of the *C. albicans TDH3* promoter (Tielker et al., 2009). In addition, plasmids expressing three or four tandem Ca*FbFP* reading frames from *TDH3p* were constructed and transformed into *C. albicans* strain CAI4; a transformant carrying empty vector pIE5 was treated similarly and served as negative control strain. Transformants were analyzed by immunoblotting as in (a) except that for strain CAI4 [pIE5-1] 300 μg of cell extract protein were used and exposure time was prolonged to allow detection of CaFbFP on the same membrane. The production of the fusions was verified and demonstrated that the amount of 2×CaFbFP was higher than that of the single protein in *C. albicans* and corresponded approximately to the 4× variant (considering that the increased signal was due to increased antibody binding sites in the 4× variant); in contrast, the amount of 3×CaFbFP was lower than both 2× and 4× variants (Fig. 1b, i). In *C. albicans*, the weak signal of 1×CaFbFP compared to multimeric CaFbFP may be due to fewer antibody binding sites but also to its enhanced degradation. The fluorescence measurements reflected the deduced CaFbFP amounts (Fig. 1b, ii). Thus, fluorescence of 1×CaFbFP (0.96 FU) was only slightly brighter than of the control strain (0.7 FU), whereas 2×CaFbFP (5.45 FU) exhibited five times higher fluorescence (Fig. 1b, ii). Interestingly, other that in *S. cerevisiae*, in *C. albicans* the 4×CaFbFP variant showed the brightest signal (6.11 FU), while fluorescence of 3×CaFbFP was stronger compared to 1×CaFbFP but about three times lower than

the intensity of 4×CaFbFP. The high fluorescence activity of 4×CaFbFP was also apparent in single colonies and streaks grown on SD agar (Fig. 1b, iii); furthermore, fluorescence was easily detectable in single cells of transformants producing 4×CaFbFP and with decreased intensity also in cells producing 2×CaFbFP (Fig. 1b, iv).

Collectively, these results suggest that double and threefold fusions of CaFbFP provide the brightest fluorescence in the cytoplasm of *S. cerevisiae*, while the fourfold fusion could further enhance CaFbFP fluorescence intensity in *C. albicans*.

3.2. Targeting CaFbFP to nucleus and cell wall of S. cerevisiae

FbFP fluorescence requires the binding of cofactors including FMN, FAD or riboflavin (Drepper et al., 2013), which may not be present at sufficient levels in cell compartments outside the cytoplasm. Therefore, targeting of FbFP to the nucleus and the cell wall of *S. cerevisiae* and its function in these locations was examined. To achieve nuclear localization, expression plasmids pIE3-HTB1 and pIE3-2-HTB1 were constructed that encode fusions of CaFbFP or 2×CaFbFP, respectively, to a resident nuclear protein, histone H2B (Kolodrubetz et al., 1982) (Fig. 2a, i). Transformants of *S. cerevisiae* strain THY.AP4 were tested by immunoblotting of crude extracts using anti-EcFbFP antiserum. The blot revealed 29.9 kDa (45.6 kDa) bands for CaFbFP (2×CaFbFP), which were absent in control transformant THY.AP4[p426GAL1] (Fig. 2a, ii). To verify nuclear localization of CaFbFP single cells were examined by fluorescence microscopy using filters for excitation with blue light (BP470/40) and detection of emission in the range of 500–550 nm. Cells producing the ScH2B-2×CaFbFP fusion showed a distinct intracellular fluorescent signal, which was shown to coincide with DAPI fluorescence indicating nuclear localization (Fig. 2a, iii); a similar but less intense fluorescence was observed also for the ScH2B-CaFbFP fusion (data not shown). In contrast, as expected cells producing non-fused CaFbFP encoded by expression vector pIE3 resulted in cytoplasmic fluorescence, while no signal was apparent in negative control cells carrying empty vector p426GAL1.

It is yet unknown if CaFbFP can be secreted, become attached to the fungal cell wall and function at this location. This idea was tested by constructing expression vectors producing a fusion of the Aga2 agglutinin to CaFbFP. In *S. cerevisiae MATa* strains, Aga2 is attached to the cell wall by disulfide-bonding to the Aga1 protein, which is bound to β1,6-glucan in the cell wall via the remnant of a glycosylphosphatidylinositol anchor (Cappellaro et al., 1994; Lu et al., 1995). Expression vector pIE9-2μ was constructed as derivative of the commercial pYD1 plasmid (Invitrogen) encoding an Aga2-CaFbFP fusion, in which CaFbFP is flanked by Xpress, V5 and 6×His epitopes (Fig. 2b, i). In transformants of strain EBY100 carrying pIE9-2μ the synthesis of the fusion protein was detectable by both immunoblotting and fluorescence microscopy. Immunoblotting using anti-EcFbFP revealed a fuzzy main band of 40–55 kDa, a mass that is considerably greater as expected from the theoretical mass of the secretion precursor (31.2 kDa); several immunoreactive proteins with lower masses were still present in transformants carrying plasmid pIE9K18A-2μ, encoding a mutein lacking the K17 K18 motif suggesting that the Kex2 protease (Fuller et al., 1989) does not degrade the fusion protein at this site (Fig. 2b, ii). The increased mass of the fusion protein is probably due to its high *O*-glycosylation, which occurs for Aga2 during secretion (Cappellaro et al., 1991). Fluorescence microscopy of pIE9-2μ transformant cells also revealed the Aga2-CaFbFP fusion as fluorescent patches on the fungal cell surface, consistent with its attachment to the cell wall (Fig. 2b, iii). It should be noted that the detection of the Aga2-CaFbFP fusion was only obtained after extensive modification of the original pYD1 plasmid, by exchang-

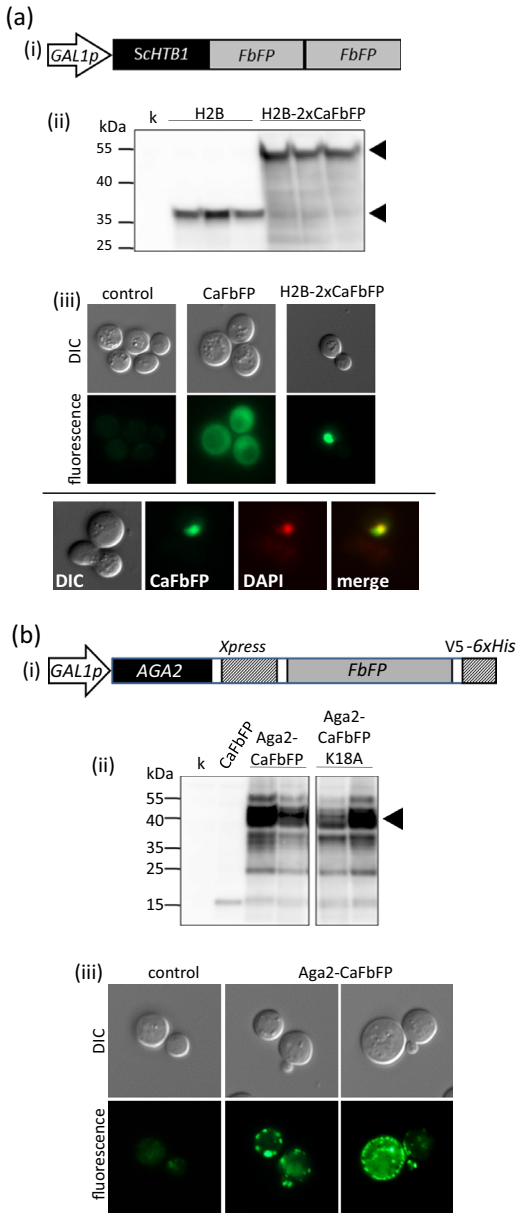

Fig. 2. Nuclear and surface localization of FbFP in *S. cerevisiae*. (a) Nuclear localization. (i) Scheme of expression units in plasmids pIE3-HTB1 and pIE3-2-HTB1 encoding a fusion of histone H2B (*HTB1* gene product) to single or two tandem FbFP proteins. (ii) Transformants of *S. cerevisiae* strain THY.AP4 were obtained and CaFbFP was detected in crude extracts (100 μg protein) by SDS-PAGE followed by immunoblot analysis using anti-EcFbFP antiserum. A transformant carrying empty vector p426GAL1 was used as control (k). (iii) Transformants were photographed under white light using differential interference contrast (DIC) imaging or excited for fluorescence by blue light (Zeiss filter set FS38HE) using an Axio Imager.M1 microscope. Cells carrying p426GAL1 (control) or pIE3 (CaFbFP in cytoplasm) were used as controls. Bottom: DIC image and fluorescence signals derived from FbFP (green) or nuclear DAPI staining (red) of a pIE3-2-HTB1 transformant (H2B-2×CaFbFP) were compared (merged yellow signal). Photographs of DAPI stainings were taken after fluorescence excitation using Zeiss filter set FS02 (excitation: G365, emission: LP420). (b) Cell wall localization of CaFbFP mediated by fusion of CaFbFP to the cell wall-associated agglutinin Aga2. (i) Scheme of the *AGA2-CaFbFP* fusion in pIE9-2μ encoding a fusion of Aga2 to FbFP and containing Xpress-, V5- and 6×His-epitopes. (ii) *S. cerevisiae* EBY100 transformants carrying pIE9-2μ (Aga2-CaFbFP) or a derivative plasmid (pIE9K18A-2μ) deleted for a putative Kex2 cleavage sequence (Aga2-CaFbFP K18A) were analyzed by immunoblotting for the presence of FbFP as in a); transformants carrying control plasmids pYD1-2μ (control) or pIE3 (CaFbFP) were used as controls. (iii) Transformants carrying pIE9-2μ (Aga2-CaFbFP) or empty plasmid pYD1-2μ (control) were analyzed for the presence of FbFP by fluorescence microscopy as in a). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ing the *CEN6/ARS4* region with the origin of replication of the 2µ plasmid (2 µm-*ori*) that generates high plasmid copy numbers. Without this modification, the fusion protein was undetectable in transformants, although immunofluorescence microscopy using rabbit anti-EcFbFP antiserum detected by FITC-labeled anti-rabbit antibody demonstrated the location of Aga2-CaFbFP as patches on the yeast cell surface (data not shown).

It can be concluded that CaFbFP can be targeted to both nucleus and cell wall of *S. cerevisiae*; furthermore, levels of FMN-type cofactors are sufficient in these localizations to show its fluorescent function. In additional experiments, we could also show that medium completely lacking riboflavin also supported fluorescence of cell wall-bound CaFbFP protein (data not shown) suggesting that loading with the cofactor occurs intracellularly during transit of the precursor to the cell surface.

3.3. Targeting CaFbFP to the nucleus in *C. albicans*

The above results had shown that CaFbFP is produced efficiently in the cytoplasm of *C. albicans*, if a tandem fusion of 2 and 4 *CaFbFP* coding regions is expressed (Fig. 1). To explore if CaFbFP can be targeted to the nucleus a similar approach as for *S. cerevisiae* (Fig. 2) was used, by fusing the 2×Ca*FbFP* and 4×Ca*FbFP* tandem reading frame, respectively, to the coding region of the *C. albicans HTB1* gene encoding histone H2B. For this purpose plasmids pIE5-2-HTB1 and pIE5-4-HTB1 were constructed, in which the histone-FbFP fusions are transcribed by the strong Ca*TDH3* promoter (Fig. 3a). Production of the fusion proteins in transformants of *C. albicans* strain CAI4 carrying pIE5-2-HTB1 or pIE5-4-HTB1 was verified by immunoblotting of crude extracts using anti-EcFbFP antiserum. The fusion proteins were represented as bands at 54 kDa and 85 kDa, respectively, which migrated slower than expected (theoretical masses H2B-2×CaFbFP: 45.9 kDa, H2B-4×CaFbFP: 77.5 kDa); the unfused proteins in strains CAI4[pIE5-2] and CAI4 [pIE5-4] migrated with an apparent mass of about 37 kDa (2×CaFbFP) and 67 kDa (4×CaFbFP), as expected (Fig. 3b). Fluorescence microscopy of single cells producing either H2B-2×CaFbFP or H2B-4×CaFbFP revealed CaFbFP fluorescence enriched in the nucleus, which coincided with DAPI staining (Fig. 3c). Confirming the location of unbound CaFbFP variants in the cytoplasm of *C. albicans* cells, fusion of histone H2B with the fourfold CaFbFP protein revealed a higher signal in the cell nuclei compared to the double variant and improved microscopic detection of CaFbFP. The experiments show that in *C. albicans*, as in *S. cerevisiae*, CaFbFP can be directed to the nucleus in a functional form to act as a fluorescent sentinel.

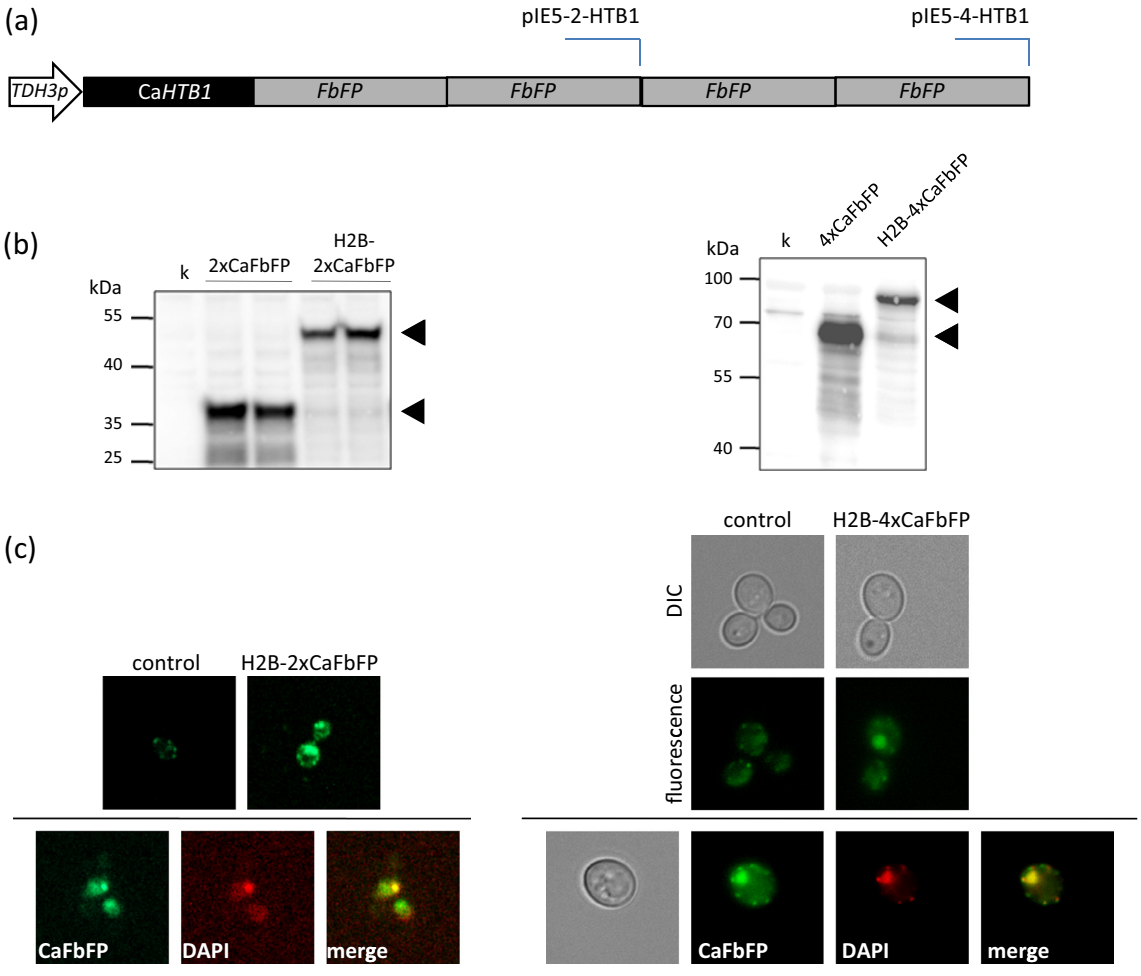

Fig. 3. Nuclear localization of CaFbFP in *C. albicans*. (a) Scheme of expression unit in pIE5-2-HTB1 and pIE5-4-HTB1 consisting of a fusion of 2×CaFbFP or 4×CaFbFP to histone H2B (*HTB1* gene product) from *C. albicans*. (b) Immunoblot analysis. Crude extracts of *C. albicans* CAI4 transformants (100 µg protein) carrying plasmids pIE5-2 (2×CaFbFP), pIE5-4 (4×CaFbFP), pIE5-2-HTB1 (H2B-2×CaFbFP) or pIE5-4-HTB1 (H2B-4×CaFbFP) were analyzed by immunoblotting using anti-EcFbFP antibodies, as described in Fig. 2. (c) Fluorescence microscopy. Left: strain CAI4 transformants carrying pIE5-2-HTB1 (H2B-2×CaFbFP) or pIE5 (control) were observed in a Cell Observer SD microscope (Zeiss) using an argon laser to generate excitation at 488 nm (CaFbFP) or 353 nm (DAPI); fluorescence signals emitted by CaFbFP or DAPI were compared as in Fig. 2. Right: transformants of strain CAI4 carrying pIE5-4-HTB1 (H2B-4×CaFbFP) or pIE5 (control) were photographed as in Fig. 2.

3.4. Biosynthesis of a CaFbFP-based O_2 sensor in S. cerevisiae

Recently, a fusion consisting of the oxygen-insensitive FbFP to the oxygen-dependent YFP fluorescent proteins (designated FluBO) has been shown to function as an *in vivo* oxygen sensor in *E. coli* (Potzkei et al., 2012). In this fusion, EcFbFP serves as donor and YFP as acceptor chromophore; following excitation of EcFbFP under normoxia, FbFP-type emission is transferred to YFP by Förster Resonance Energy Transfer (FRET), while under hypoxia, FbFP-type fluorescence is detected (Potzkei et al., 2012). To assess if a similar type of fusion protein can be produced in fungi we constructed expression plasmids for cytoplasmic production (pOXY2) and cell wall localization (pOXY4) of a YFP-FbFP fusion in *S. cerevisiae*. For this purpose, a *YFP* variant gene optimized for the expression in *C. albicans* (Gerami-Nejad et al., 2001) was fused to the Ca*FbFP* gene in such a way that both encoded proteins are connected by a peptide of 14 amino acids, as has been done for the FluBO protein (Potzkei et al., 2012); however, to allow correct translation of the fusion also in *C. albicans* the fusion gene was altered in its linker sequence to replace the CUG codon (encoding serine in *C. albicans*; (Santos et al., 1993) to the universal UUG codon for leucine (Fig. 4a). The YFP-CaFbFP fusion gene was either placed directly downstream of the *GAL1* promoter (pOXY2) or by first extending it by addition of *AGA2* sequences (pOXY4) in order

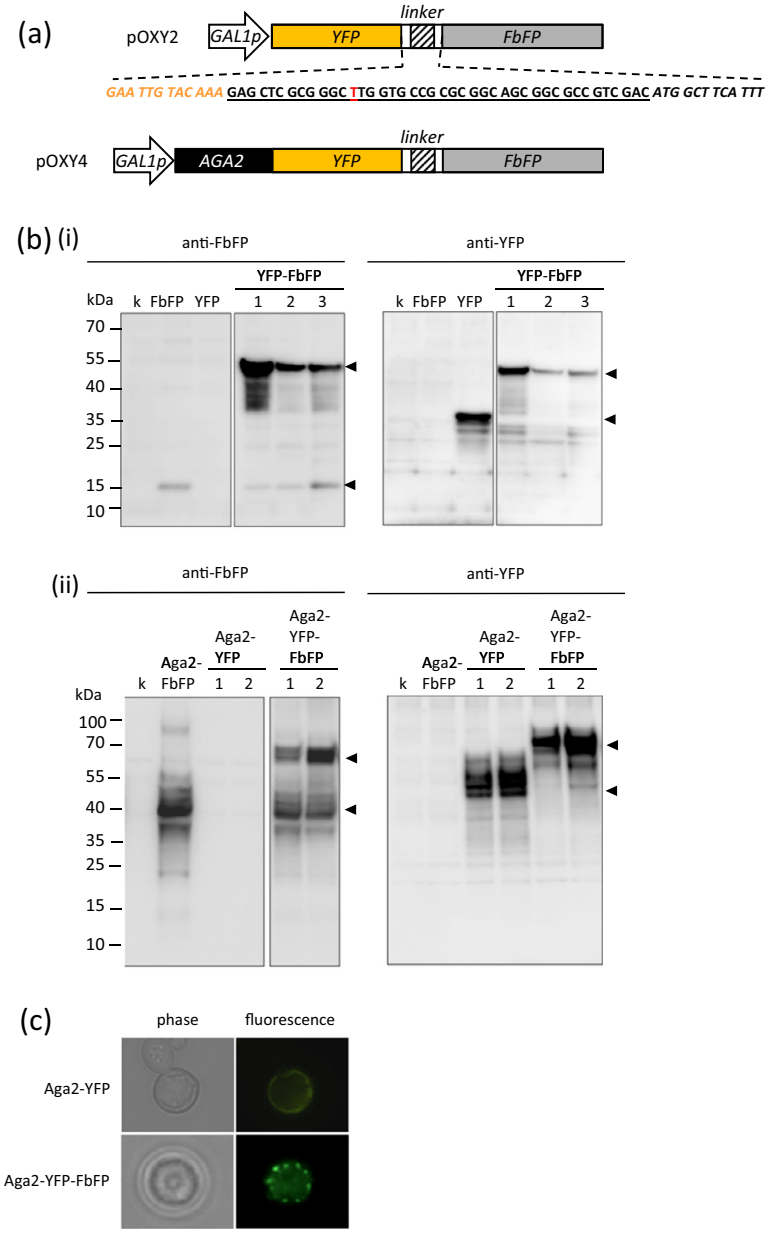

Fig. 4. Production of YFP-FbFP fusions in *S. cerevisiae*. (a) Scheme of expression plasmids. In pOXY2 codon-optimized *YFP* (Gerami-Nejad et al., 2001) is fused to Ca*FbFP* via a 42 bp-linker sequence; the fusion is transcribed by the *GAL1* promoter. pOXY4 contains *AGA2* joined to the *YFP-FbFP* fusion. (b) Immunoblots demonstrating the YFP-FbFP fusion in transformants. Crude extracts (100 µg protein) of transformants were separated by SDS-PAGE (4–20% acrylamide) followed by immunoblotting using anti-EcFbFP or anti-GFP/YFP antibodies. The migration of FbFP and YFP proteins is indicated by arrows. (i) *S. cerevisiae* THY.AP4 transformants carrying pOXY2 (YFP-FbFP), pOXY1 (YFP), pIE3 (FbFP) or p426GAL1 (k) were analyzed. (ii) *S. cerevisiae* EBY100 carrying pOXY4 (Aga2-YFP-FbFP), pOXY3 (Aga2-YFP), pIE9-2µ (Aga2-FbFP) or p426GAL1 (k) were analyzed. (c) Cells of *S. cerevisiae* EBY100 transformants carrying pOXY3 (Aga2-YFP) or pOXY4 (Aga2-YFP-CaFbFP) were analyzed by phase contrast imaging and fluorescence microscopy during excitation with green (Aga2-YFP) or blue (Aga2-YFP-CaFbFP) light using Zeiss filter set FS46 (excitation: BP500/20, emission: BP535/30) or FS38HE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to direct the encoded Aga2-YFP-CaFbFP fusion protein to the cell wall (Fig. 4a).

The synthesis of the YFP-CaFbFP fusion was verified by immunoblotting using either anti-GFP/YFP antibody or anti-EcFbFP antibody for detection in crude extracts of transfomants. As controls, extracts of transformants solely producing YFP including the linker peptide (pOXY1) or CaFbFP (pIE3) were tested. For pOXY2 transformants, the expected mass (theoretical mass 43.9 kDa) of the fusion protein could be detected with both antibodies (Fig. 4b, i). In addition, a minor band of about 15 kDa was found to react with anti-EcFbFP but not the anti-YFP antibody suggesting partial cleavage of the fusion in the linker peptide. For pOXY4 transformants, crude extracts of *S. cerevisiae* strain EBY100 transformants producing Aga2-CaFbFP (pIE9-2μ), Aga2-YFP (pOXY3), Aga2-YFP-CaFbFP (pOXY4) or no fluorescent protein (pYD1-2μ) were examined by immunoblotting. A protein reacting with both antibodies of about 60 kDa was detected that represents the Aga2-YFP-CaFbFP fusion protein, at an apparent greater mass as expected from its theoretical mass of 59.4 kDa (Fig. 4b, ii); this abnormality may be due to *O*-glycosylation of the Aga2 moiety (as for the pIE9-2μ transformants, see above). As for pOXY2 transformants, a protein only reacting with anti-EcFbFP antibody was detected at about equal levels to the full-length fusion protein, which very likely arose by proteolytical removal of the YFP moiety.

Using fluorescence microscopy, the cytoplasmic localization of the YFP-CaFbFP fusion could be verified as for the unfused CaFbFP protein (data not shown). Cell wall localization of the Aga2-YFP-FbFP fusion protein was confirmed by showing its fluorescence on the surface of pOXY4 transformants (Fig. 4c); as in the case of the Aga2-CaFbFP fusion (Fig. 2b, iii) the protein was not distributed evenly but occurred in patches. The Aga2-YFP fusion produced by the pOXY3 transformant also presented surface fluorescence.

3.5. The YFP-CaFbFP fusion (YFOS) acts as an oxygen sensor in S. cerevisiae

Successful production of the YFP-CaFbFP fusion in the cytoplasm or at the cell surface allowed testing of its O_2 sensor function, which based on FRET between both fluorescent proteins (Potzkei et al., 2012). A scheme demonstrating FRET in the presence of oxygen is shown in Fig. 5a; the indicated wavelengths were used or determined experimentally in this study. First, FRET was assessed for whole cells of strain THY.AP4 carrying plasmid pOXY2 for cytoplasmic production, using as controls cells producing no or a single fluorescent protein. In these experiments, an excitation wavelength of 380 nm was used, which mainly excites FbFP but not YFP (Potzkei et al., 2012). Under normoxia, a transformant only producing CaFbFP (pIE3) showed an emission spectrum with a maximum in the range of 500–505 nm, close to the previously determined emission maximum of 495 nm in crude extracts (Tielker et al., 2009). In contrast, the YFP-CaFbFP producing transformant (pOXY2) showed an emission maximum at 530 nm, which is characteristic for YFP fluorescence (em max 527 nm), whereas control cells producing solely YFP did not emit light if excited at 380 nm (Fig. 5b) but showed fluorescence if excited at 500 nm, as expected (data not shown). These experiments indicate clearly that the YFP-CaFbFP fusion protein shows FRET that depends on the presence of oxygen to mature YFP. Because the coding region for the fungal-adapted YFP-CaFbFP fusion differs significantly from the previously described FluBO fusion gene (Potzkei et al., 2012) it is referred to in the following as YFOS (“yeast fluorescent oxygen sensor”). In supplemental experiments we compared the pH-dependence of FRET fluorescence by YFOS with fluorescence of CaFbFP and YFP, by incubating crude extracts of the respective transformants in aerated buffers of varying pH values (3.5–8.8). The results indicate that fluorescence of YFOS and YFP have similar pH dependencies, by showing steeply increasing levels at pH $\geqslant$ 6.5; in contrast, CaFbFP fluorescence was less dependent on pH values and occurred at $\geqslant$pH 5 (Fig. S1).

Next, the ability of the YFOS protein to respond to different oxygen conditions was determined. Emission spectra were recorded of whole cells grown under 10, 4 and 0.2% oxygen (Fig. 5b). The results showed that with depletion of oxygen, the emission spectrum changed from a YFP-like spectrum under normoxia to a CaFbFP-like spectrum under highly hypoxic conditions (0.2% O_2). The ratio of fluorescence intensity at 530 nm (YFP-mediated) to 500–505 nm (CaFbFP-mediated), suggested a linear correlation against oxygen concentrations (Fig. 5c). Thus, cytoplasmic YFOS protein appears to act as whole cell oxygen sensor in *S. cerevisiae*.

Furthermore, we tested the ability of YFOS to serve as an oxygen sensor by detecting the decreasing oxygen conditions during yeast growth under suboptimal oxygen conditions. For this purpose we performed fluorescence analyses of the YFOS-producing *S. cerevisiae* strain THY.AP4[pOXY2] grown in shake flasks sealed airtight to prevent oxygen exchange; probes were taken during exponential growth phase (320 min) and stationary phase (24 h). Fluorescence measurements clearly revealed hypoxic conditions in cell cultures of the stationary phase cells as indicated by shift of the YFOS emission spectrum, in which the YFP to CaFbFP fluorescence ratio was decreased; in contrast, only a minor shift was detected in exponentially-growing cells (Fig. 5d).

To also assess sensor properties of the YFOS protein fused to the Aga2 protein in the yeast cell wall, emission spectra of *S. cerevisiae* EBY100 cells carrying plasmid pOXY4 (Aga2-YFOS; scheme in Fig. 5e, top) were recorded under normoxic and hypoxic (0.2% O_2) conditions. In these experiments, an excitation wavelength of 430 nm was used to improve the excitation energy for FbFP. Cells grown under normoxia showed an emission maximum at 525 nm consistent with YFP emission, while control cells only producing Aga2-CaFbFP (pIE9-2μ transformant) showed a typical CaFbFP spectrum and cells containing Aga2-YFP (pOXY3 transformant) did not fluoresce (Fig. 5e, bottom); however, the Aga2-YFP producer showed fluorescence if excited at 500 nm, proving that in principle, YFP within this fusion and at the cell surface is produced in a functional form (data not shown). After growth under hypoxic conditions emission of cells with Aga2-CaFbFP and Aga2-YFP behave as recorded under normoxic conditions (Fig. 5e, bottom). In contrast, a weaker YFP signal compared to normoxic conditions could be observed for the spectrum of cells containing Aga2-YFOS, resulting in an increase of the YFP-mediated (525 nm) to CaFbFP-mediated (500 nm) fluorescence ratio at rising oxygen concentration (normoxia: 1.05, hypoxia: 0.88). Thus, these results indicate that surface-bound YFOS can also be used as an oxygen sensor in live yeast cells.

3.6. FRET activity of YFOS in C. albicans

To study the functionality of the FRET system in *C. albicans*, we constructed several expression plasmids for YFP-CaFbFP fusions, in which the Ca*FbFP* reading frame occured once (pOXY8 encoding YFOS-1), twice (pOXY9 encoding YFOS-2) or four-fold (pOXY11 encoding YFOS-4) (Fig. 6a); as a control vector pOXY7 encoding solely *YFP* with the linker sequence was used. In transformants of *C. albicans* CAI4 with these plasmids the synthesis of all three fusion proteins could be verified by immunoblotting, which reacted with both anti-EcFbFP and anti-GFP/YFP antisera (Fig. 6b). This experiment also showed that the protein amount of YFOS-1 was relatively low in contrast to the two other variants; highest amounts were obtained for the YFOS-2 protein. Regarding the positive controls, YFP showed a weak signal at about 28 kDa (theoretical mass 28.3 kDa) in immunoblots using anti-GFP/YFP antiserum. 2×CaFbFP could be detected as a clear band at the

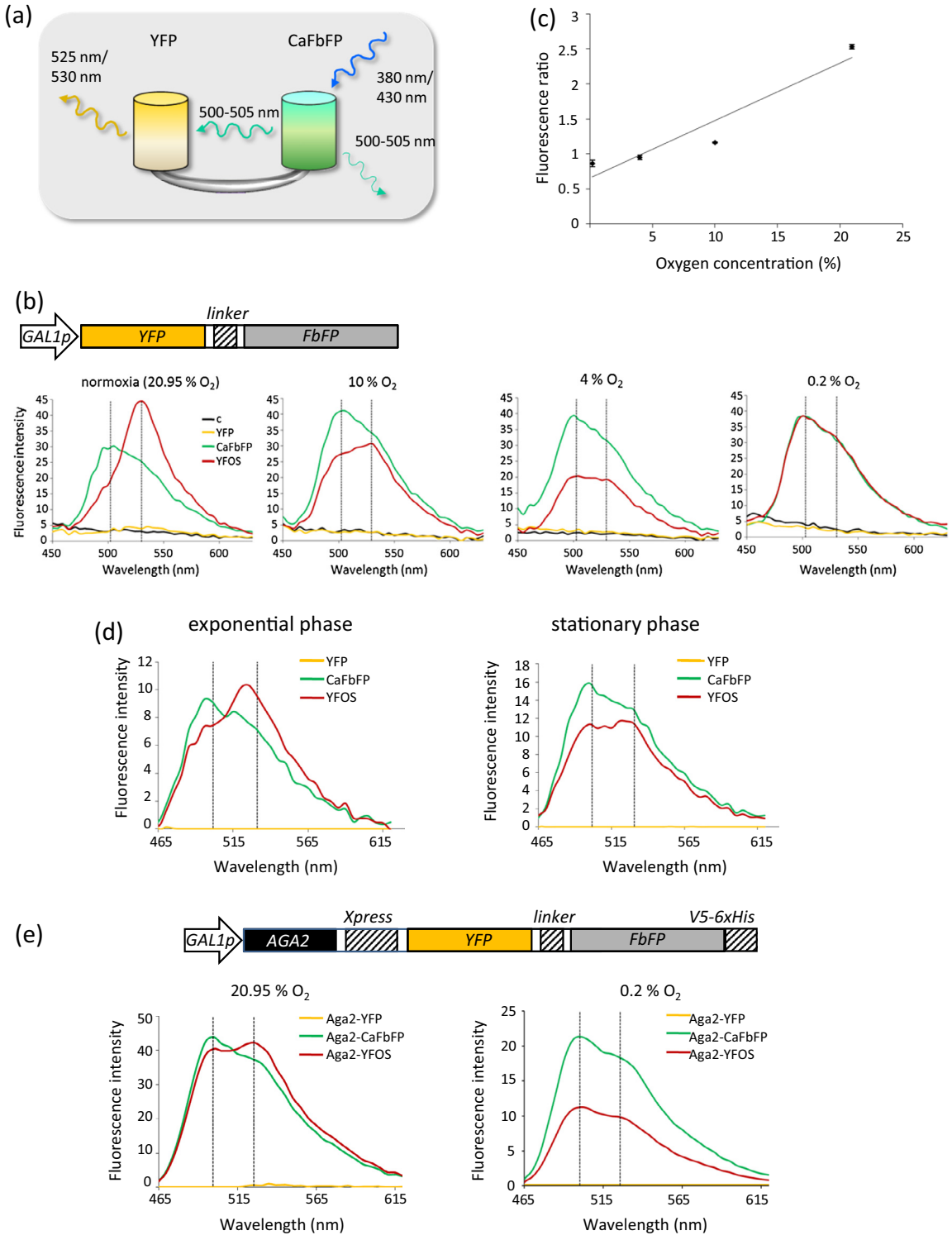

Fig. 5. CaFbFP as FRET donor for YFP in *S. cerevisiae*. (a) Principle of FRET in YFP-FbFP fusion (YFOS). FbFP is excited at 380 or 430 nm and emits light at 500–505 nm (whole cells) that excites YFP, which is apparent by emitted light of 530 nm wavelength (525 nm for surface display). (b) Top: scheme of YFOS fusion encoded by plasmid pOXY2. Bottom: fluorescence profiles of *S. cerevisiae* THY.AP4 transformants (plasmids) producing YFOS (pOXY2), YFP (pOXY1), CaFbFP (pIE3) or no fluorescent protein (p426GAL1). Cells were grown under normoxic conditions (20.95% O_2) or under hypoxia (10, 4 or 0.2% O_2) and fluorescence emission (λ_{exc} = 380 nm) of equal numbers of cells was assessed using a Tecan infinite M200 fluorometer. Dotted lines mark emission maxima of CaFbFP (500–505 nm) and YFP (530 nm) in whole cells. (c) Standard curve to determine intracellular oxygen concentrations through YFOS-mediated fluorescence. X-axis: percentage of oxygen concentration, Y-axis: ratio of YFP (530 nm) to CaFbFP (average of 500 and 505 nm) fluorescence intensity of YFOS. Data calculated and standard deviation of the means of three technical replicates derived from (b) (deviation < 5% of mean values) and plotted by a linear regression line. (d) Use of YFOS for determination of decreasing oxygen conditions during yeast growth. Liquid cultures of *S. cerevisiae* THY.AP4 strains expressing *YFOS* (pOXY2), *YFP* (pOXY1), Ca*FbFP* (pIE3) or the empty plasmid (p426GAL1) were grown in airtight closed shake flasks. After 320 min (exponential phase) and 24 h (stationary phase) fluorescence emission was verified analogous to (b). (e) Emission spectra (λ_{exc} = 430 nm) of whole cells of *S. cerevisiae* EBY100 transformants carrying plasmid pOXY4 (Aga2-YFOS), pOXY3 (Aga2-YFP) or pIE9-2μ (Aga2-CaFbFP) grown under normoxic or hypoxic (0.2% O_2) conditions; values for control cells of *S. cerevisiae* EBY100 carrying control plasmid pYD1-2μ were used for background correction.

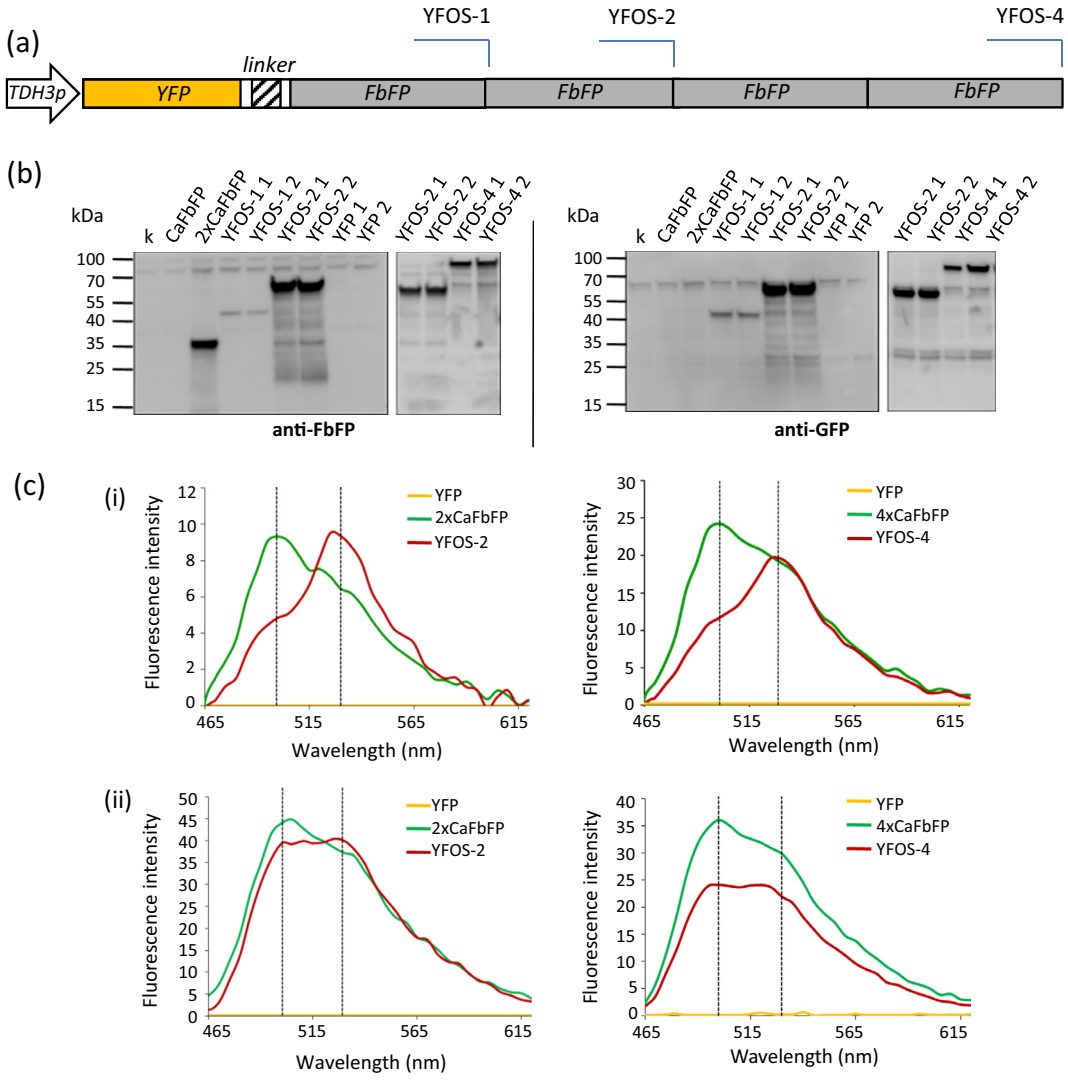

Fig. 6. CaFbFP as FRET donor for YFP in *C. albicans*. (a) Scheme of YFOS fusions. Plasmids encoded YFP-fusions containing one copy (pOXY8), two copies (pOXY9) or four copies (pOXY11) of the FbFP reading frame. (b) Immunoblots of crude extracts (100 μg protein) from transformants of strain CAI4 carrying pOXY plasmids or control plasmids pIE5 (k), pIE5-1 (CaFbFP) or pIE5-2 (2×CaFbFP). (c) Emission spectra (λ_{exc} = 430 nm) of whole cells of *C. albicans* CAI4 transformants carrying plasmids pOXY9 (YFOS-2), pOXY11 (YFOS-4), pOXY7 (YFP), pIE5-2 (2×CaFbFP) or pIE5-4 (4×CaFbFP) grown under normoxia (i) or hypoxia (0.2% oxygen) (ii).

expected height, while the single CaFbFP was not detectable because of low protein amounts (Fig. 1b, i). Under normoxia, the emission spectrum of the YFOS-2 and YFOS-4 transformant revealed the successful energy transfer between YFP and 2×CaFbFP and 4×CaFbFP, respectively (Fig. 6c, i); this result was also obtained at lower levels for YFOS containing 1×CaFbFP (data not shown). We note, however, that FRET efficiency was slightly higher for YFOS-2 (1.94) as compared to YFOS-4 (1.69). For these experiments with *C. albicans*, an excitation wavelength of 430 nm was found to be optimal to achieve FRET. If the transformants producing YFOS-2 and YFOS-4 were grown under hypoxia (0.2% O_2) different spectra as under normoxia were obtained for whole cells. In this condition, YFP emission at 530 nm was greatly reduced (YFP/CaFbFP fluorescence ratio YFOS-2: 0.97, YFOS-4: 0.91) indicating low FRET activity (Fig. 6c, ii). Thus, it appears that the YFOS-2 and YFOS-4 fusions act as oxygen sensors in *C. albicans*.

4. Discussion

As fluorescent reporters, FbFPs outperform GFP and its variants in several aspects because of their small size (Chapman et al., 2008), oxygen-independent fluorescence (Drepper et al., 2013), enhanced thermal stability, rapid maturation of fluorescence and because of their broad pH range of activity (Mukherjee et al., 2013). With regard to their oxygen-independent activity, FbFPs were found to be applicable as a fluorescent marker and gene reporter protein for gastrointestinal anaerobic symbionts including *Bifidobacterium* and *Bacteroides* species (Landete et al., 2014; Lobo et al., 2011). For *E. coli*, EcFbFP was established as a preferred reporter of growth in normoxic cultures at high-density (Drepper et al., 2007, 2010) or to monitor conjugation under anoxic growth (Król et al., 2010). FbFPs have also been used as reporters of gene expression in mammalian cell lines, as well as in murine and neuronal stem cells (Walter et al., 2012). In addition, we have previously shown that codon-optimized EcFbFP (CaFbFP) functions as reporter protein in the cytoplasm of the fungi *S. cerevisiae* and *C. albicans* (Tielker et al., 2009). Here we extend these findings by demonstrating that in fungi, CaFbFP has activity in other cellular compartments and can be used as a component of an *in vivo* oxygen sensor.

We report that fusions to the histone H2B lead to quantitative nuclear localization of CaFbFP into the nucleus of both *S. cerevisiae* and *C. albicans*. The construction of these fusions containing tan-

dem CaFbFP repeats was guided by preparatory studies to determine the effects of CaFbFP multimerization its production and fluorescence in the cytoplasm. In both fungi both parameters were found to increase significantly by producing a tandem (2×) CaFbFP repeat, which was further slightly enhanced for a 4× fusion in *C. albicans*, but not in *S. cerevisiae*. Interestingly, in *S. cerevisiae* a 3× fusion increased CaFbFP production/fluorescence, while in *C. albicans* it appeared less active than the 2× and 4× fusions suggesting that folding and/or degradation of the 3× fusion differ in both fungi. Therefore, variants with the smallest possible mass while allowing sufficient fluorescence micrsocopical detection appeared most suitable to compare nuclear import in fungi; this concept was realized by histone fusions to the single and double CaFbFP protein in *S. cerevisiae* and double and fourfold tandem versions in *C. albicans*, respectively. In addition to its nuclear localization we also obtained evidence for production and activity of CaFbFP on the cell wall of *S. cerevisiae*. This was achieved by fusion to the Aga2 agglutinin, which binds the Aga1 protein that is linked to β1,6-glucan through the remnant of a GPI-anchor in the cell wall (Cappellaro et al., 1994). A patch-like distribution of Aga2-CaFbFP on the surface was shown, which may be due to FbFP dimer formation (Drepper et al., 2013). Strong CaFbFP fluorescence observed on the cell wall and in the nucleus raises questions regarding the origin of the flavin cofactor in these localizations. It is known that heterologous production of LOV domains results in mixtures of proteins containing all flavins, i.e. riboflavin, FMN and FAD (Laan et al., 2004); other studies have shown that all flavins result in functional FbFP proteins (Mansurova et al., 2011; Dorn et al., 2013). Although both *S. cerevisiae* and *C. albicans* synthesize riboflavin, it can also be imported from the medium (Abbas and Sibirny, 2011). It is also of interest that in flavogenetic yeasts, transcriptional repression of riboflavin synthesis is exerted by iron ions and not by flavins and that high amounts of riboflavin are secreted into the culture fluid under low iron conditions (Abbas and Sibirny, 2011). Thus, in these conditions the application of FbFPs as reporter proteins may be limited by high fluorescent background levels due to the increased levels of riboflavin in the cytoplasm and the medium. Collectively, these results suggest that the yet undefined flavin cofactor bound by cell wall-bound CaFbFP in *S. cerevisiae* may be derived either from flavins present either in the external medium (excreted or as component of semisynthetic growth medium) or in the secretory pathway. We found that the omission of riboflavin in the standard semisynthetic yeast medium used to support yeast growth (yeast nitrogen base) did not reduce CaFbFP fluorescence, which could be due to the presence of flavins and maturation of CaFbFP in compartments of the classical secretion pathway.

FbFPs have found applications as biosensors, i. e. to measure copper (Ravikumar et al., 2015), pH (Liu et al., 2014) and molecular oxygen (Potzkei et al., 2012). To realize *in vivo* O_2 measurements, a fusion of oxygen dependent YFP to oxygen-independent EcFbFP was produced in *E. coli*. Excitation of FbFP fluorescence yielded YFP-type of emission under normoxia by FRET but FbFP-type of emission under hypoxia. This system was able to monitor temporal changes of O_2 levels in the cytoplasm of *E. coli* (Potzkei et al., 2012). Based on this idea we generated *S. cerevisiae* and *C. albicans* strains producing fusions of YFP to CaFbFP (designated YFOS) in their cytoplasms. YFOS fusions were produced efficiently in both fungi, although in the case of *C. albicans* only a 2× (YFOS-2) or 4× (YFOS-4) tandem CaFbFP moiety in the fusion generated sufficient amounts of protein. YFOS-2 turned out to be most suitable as oxygen sensor in *C. albicans*, as it exhibits a smaller size, higher protein amount in the cell and mainly a higher FRET efficiency compared to YFOS-4. Moreover, emission spectra obtained from whole cells of *S. cerevisiae* transformants producing YFOS or *C. albicans* cells with YFOS-2 or YFOS-4 demonstrated clear spectral differences consistent with FRET occuring under normoxia but not under hypoxia. In agreement with the findings of Potzkei et al. (2012), a gradual spectral shift correlating with oxygen concentrations was found demonstrating the ability of YFOS to reflect O_2 levels in the yeast cytoplasm. Accordingly, the use of YFOS as oxygen sensor in growing yeast cultures enabled the record of decreasing oxygen concentrations during growth of *S. cerevisiae*. Plots of the FbFP/YFP-ratio versus different oxygen concentrations yielded a linear relationship in first approximation. However, additional work is required to specify the responsiveness of the YFOS sensor in the yeast cytoplasm with regard to oxygen, pH and other environmental parameters.

Interestingly, even the linkage of YFOS to the cell wall produced a functional oxygen sensor, which may be applicable to measure O_2 levels externally to cells directly, i.e. to measure oxygen levels in the culture fluid. Future work may reveal if YFOS bound to the cell wall of *C. albicans* may be used to assess oxygen concentrations occurring in its vicinity during biofilm formation, within human cells and tissues and in organs with varying oxygen levels.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.04.004.

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