Click beetle luciferases as dual reporters of gene expression in *Candida albicans*

Mario Kapitan, Isabel Eichhof, Quentin Lagadec and Joachim F. Ernst

Department Biologie, Molekulare Mykologie, Heinrich-Heine-Universität Düsseldorf, 1/26 12, Duesseldorf, D-40225, Germany

Synthetic genes encoding functional luciferases of the click beetle (CB) *Pyrophorus plagiophthalamus* have been expressed in the human fungal pathogen *Candida albicans*. Both green- and red-emitting CB luciferases (CaCBLuc and CaCBRluc) were produced with high efficiency in transformants under transcriptional control of the growth-dependent ACT1 promoter, as well as by the HWP1 and UME6 promoters, which are upregulated during hyphal morphogenesis, as well as by the YWP1 and EFG1 promoters, which are downregulated. For all hyphally regulated genes, relative bioluminescence values derived from promoter fusions approximated relative transcript levels of native genes, although downregulation of YWP1 promoter activity required correction for the stability of CB luciferases (approximate half-lives 30 min for CaCBLuc and 80 min for CaCBLuc, as determined by immunoblotting). Importantly, the activity of both luciferases could be separated monitored in a single strain, in intact cells, in lysed cells or in cell extracts using luciferin as single substrate and inhibition of hypha formation by farnesol could be easily detected by the *HWP1p-CaCBLuc* fusion. The results suggest that CB luciferases are convenient tools to measure gene expression in *C. albicans* and may facilitate screenings for antifungal compounds.

**INTRODUCTION**

Transcriptional circuits govern host interaction and virulence of the human fungal pathogen *Candida albicans* (Sudbery, 2011; Fox & Nobile, 2012; Lu et al., 2014). Several heterologous reporters monitoring gene expression, which bypass its unusual codon usage of translating CUG into serine instead of leucine (Santos et al., 1993), have been developed for this fungus. The reporters include β-galactosidases, as well as light-producing fluorescent and luminescent proteins (reviewed by Papon et al., 2012). Luciferases are of special interest as reporters, because their bioluminescence does not require external light excitation, thereby avoiding phototoxic damage, bleaching and high background signals in cells. These characteristics have led to the use of luciferases for non-invasive imaging of pathogenic microbes in live animals (Doyle et al., 2006a; Enjalbert et al., 2009; Vande Velde et al., 2014b). In *C. albicans* three luciferases have been used including enzymes derived from *Renilla reniformis* and *Gaussia princeps* (RLUC, GLUC), which utilize coelenterazine as the luminescent substrate (Srikantha et al., 1996; Enjalbert et al., 2009), and the luciferase of the firefly *Photinus pyralis* (FLUC) that requires luciferin as substrate (Doyle et al., 2006b). A relatively low permeability of coelenterazine and luciferin across the *C. albicans* cell wall, especially of hyphal cells (Doyle et al., 2006a, b), was reported but surface display of GLUC by fusion to a cell wall protein remedied this problem (Enjalbert et al., 2009; Vande Velde et al., 2014a). GLUC and FLUC enzymes were successfully used to visualize fungal spread in mice in real time, although it appeared difficult to reach an even distribution of the externally applied luminescent substrate in the infected animal (Doyle et al., 2006a; Enjalbert et al., 2009; d’Enfert et al., 2010; Vande Velde et al., 2014b).

Here we describe the use of luciferases derived from the click beetle (CB) *Pyrophorus plagiophthalamus* as reporters of gene expression in *C. albicans*. These animals contain luciferases that produce light of different colours by oxidizing the substrate luciferin in the presence of ATP and oxygen (Wood et al., 1989). The original CB luciferase-encoding sequences were optimized for expression in mammalian cells by Almond et al. (2003) to generate *CBRluc* and *CBLuc* genes encoding luciferases producing well-separated red and green luminescence does not require external light excitation, thereby avoiding phototoxic damage, bleaching and high background signals in cells. These characteristics have led to the use of luciferases for non-invasive imaging of pathogenic microbes in live animals (Doyle et al., 2006a; Enjalbert et al., 2009; Vande Velde et al., 2014b). In *C. albicans* three luciferases have been used including enzymes derived from *Renilla reniformis* and *Gaussia princeps* (RLUC, GLUC), which utilize coelenterazine as the luminescent substrate (Srikantha et al., 1996; Enjalbert et al., 2009), and the luciferase of the firefly *Photinus pyralis* (FLUC) that requires luciferin as substrate (Doyle et al., 2006b). A relatively low permeability of coelenterazine and luciferin across the *C. albicans* cell wall, especially of hyphal cells (Doyle et al., 2006a, b), was reported but surface display of GLUC by fusion to a cell wall protein remedied this problem (Enjalbert et al., 2009; Vande Velde et al., 2014a). GLUC and FLUC enzymes were successfully used to visualize fungal spread in mice in real time, although it appeared difficult to reach an even distribution of the externally applied luminescent substrate in the infected animal (Doyle et al., 2006a; Enjalbert et al., 2009; d’Enfert et al., 2010; Vande Velde et al., 2014b).

**Abbreviations:** ATP, adenosine triphosphate; Ca, *Candida albicans*; CB, click beetle; FLUC, firefly luciferase; GLUC, *Gaussia princeps* luciferase; L$_{\text{max}}$, maximal luminescence value; RLU, relative light units; RLUC, *Renilla reniformis* luciferase; RTL, relative transcript levels.

Accession numbers: KU194209, KU194210.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.
green bioluminescence light (emission maxima 613 nm and 537 nm, respectively) and have been used for chromatin analyses in the fission yeast Schizosaccharomyces pombe (Shimada & Bühler, 2012). In mammalian cells the CBGluc luciferase generated a far greater light output compared to the FLUC enzyme (Miloud et al., 2007). An enhanced green-emitting luciferase derived from another CB species (ELuc9 from Pyrearinus termittilluminans) also was found to be superior to FLUC (Nakajima et al., 2010), and its combination with red-emitting Stable Lucerase Red from a railroad worm as reporters allowed simultaneous monitoring of two gene expression units in the same animal cell (Yasunaga et al., 2014). In this report we describe methods to produce strong bioluminescent light signals by CBRluc and CBGluc luciferases in the human fungal pathogen C. albicans and demonstrate their uses as dual reporters by assessing the expression of the two relevant genes in the same strain during hyphal morphogenesis, an important virulence trait of this fungus.

**METHODS**

**Strains and growth conditions.** C. albicans strains are listed in Table 1. Strains were grown in YPD or supplemented SD minimal medium as described (Sherman et al., 1986). To induce hyphae, cells were pre-grown in YPD medium, followed by resurrection in sterile water at an OD$_{600}$=0.2. After a starvation period of 1 h at 30°C, hypha formation was initiated by addition of 10% horse serum and incubation at 37°C (110 rpm). For every point of induction hyphal formation was observed by microscopy (Axioskop 40 FL; Carl Zeiss). In some experiments 150 µM farnesol was added to block hyphal formation (Mosel et al., 2005).

**Click beetle luciferase genes and chromosomal insertion.** Synthetic CBRluc and CBGluc genes (Almond et al., 2003; CBRluc accession AY258591; CBGluc accession AY258593) were re-designed for efficient expression in C. albicans using the GeneOptimizer software (Raab et al., 2010) by replacing all CUG leucine codons (Santos et al., 1993), lowering the GC content and by adapting the sequence to the codon bias index of this fungus; the synthetic genes were produced by Life Technologies GmbH (Darmstadt) and supplied as vectors CBRluc-pMK-RQ and CBGluc-pMK-RQ (accession numbers CaCBRluc: KU194209, CaCBGluc: KU194210). BamHI-BglII fragments carrying either gene were then placed next to the HIS1 selection marker by insertion into the BamHI site of pGEM-HIS1 (Wilson et al., 1999) to generate plasmids pGEM-HIS-CBR and pGEM-HIS-CBG. Alternatively, vectors were constructed by ligating either gene adjacent to the URA3 selection marker into the BamHI site of pS1044-1 (CaURA3 CaLEU2 CAR5; kindly supplied by D. Sanglard), resulting in plasmids pD-CBR and pD-CBG. These plasmids allowed PCR co-amplification of CBRluc/ CBGluc with either selection marker.

To generate transformants of C. albicans BWP17, in which CaCBGluc (CaCBRluc) is inserted into the second exon of the ACT1 coding region (Losberger & Ernst, 1989), an insertion fragment was generated by PCR with primers inACT1GU Fw/Bw (inACT1RH Fw/Bw) using plasmid pD-CBG (pGEM-HIS-CBR) as the template (all oligonucleotides are listed in Table S1, available in the online Supplementary Material). Primers contained a terminal 60 bp sequence homologous to the second ACT1 exon, which upon transformation of strain BWP17 with the insertion fragment generated Ura+ (His1+) transformants carrying CaCBGluc (CaCBRluc) under transcriptional control of the ACT1 promoter. Correct insertion of the fragment was verified by colony PCR using primers ACT1 Dia Fw/Luci Dia Bw that generated 1.9 kb fragments and were confirmed by Southern blotting (data not shown). Probes for Southern blots were amplified using the primers URA3-for/rev or HIS1 Southern Fw/Bw. The resulting verified transformant strain was named ACT1GU (ACT1RH). A scheme of the insertion in strain ACT1GU is shown in Fig. 1a. Similarly, strain ACT1RU was constructed, in which CaCBGluc was placed downstream of the ACT1 promoter. Furthermore, CaCBRluc was inserted downstream of the HWP1 promoter to generate the strain HWP1RH. In this case, the insertion cassette was amplified by PCR using primers inHWP1RH Fw/Bw and plasmid pGEM-HIS-CBR as template; correct chromosomal insertion was verified by colony PCR using primers HWP1 Dia Fw/Lucia Dia Bw generating a 716 bp fragment and subsequent Southern blotting (data not shown). To place CaCBGluc downstream of the YWP1 promoter the insertion fragment was generated by PCR using primers inYWP1GU Fw/Bw and plasmid pD-CBG as the template; correct chromosomal insertion was verified by colony hybridization using primers YWP1 Dia Fw/Lucia Dia Bw generating a 1273 bp fragment and subsequent Southern blotting (data not shown). The resulting verified transformant was named YWP1GU, strain YWP1GU/HWP1RH contains the fusion of the HWP1 promoter to CaCBGluc, which was transformed in strain YWP1GU. Furthermore, CaCBRluc and CaCBGluc were inserted downstream of the EFG1 and UME6 promoters to generate strains EFG1RH and UME6GU (UME6GU/EFG1RH). In this case, the insertion cassette was PCR amplified using primers inEFG1RH Fw/Bw (inUME6GU Fw/Bw) and

| Table 1. Strains |
|-----------------|-----------------|-----------------|
| Strains         | Genotype        | Reference       |
| BWP17           | ura3-iro1Δ::kinm434/ur3-iro1Δ::Amnm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG | Wilson et al. (1999) |
| ACT1GU          | as BWP17 but ACT1/act1:: (ACT1p-CaCBGluc URA3) | This work |
| ACT1RH          | as BWP17 but ACT1/act1:: (ACT1p-CaCBRluc HIS1) | This work |
| ACT1RU          | as BWP17 but ACT1/act1:: (ACT1p-CaCBRluc URA3) | This work |
| YWP1GU          | as BWP17 but YWP1/ywp1:: (YWP1p-CaCBGluc URA3) | This work |
| HWP1RH          | as BWP17 but HWP1/hwp1:: (HWP1p-CaCBRluc HIS1) | This work |
| EFG1RH          | as BWP17 but EFG1/efg1:: (EFG1p-CaCBRluc HIS1) | This work |
| UME6GU          | as BWP17 but UME6/ume6:: (UM6p-CaCBGluc URA3) | This work |
| YWP1GU/HWP1RH   | as BWP17 but YWP1/ywp1:: (YWP1p-CaCBGluc URA3) HWP1/hwp1:: (HWP1p-CaCBRluc HIS1) | This work |
| UME6GU/EFG1RH   | as BWP17 but UME6/ume6:: (UM6p-CaCBGluc URA3) EFG1/efg1:: (EFG1p-CaCBRluc HIS1) | This work |

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plasmid pGEM-HIS-CBR (pD-CBG) as template; correct chromosomal insertion was verified by colony PCR using primers EFG1 Dia Fw/Luci Dia Bw (UME6 Dia Fw/Luci Dia Bw), which generated a 1199 bp fragment (1095 bp), and by Southern blotting (data not shown).

**CB luciferase measurements.** To measure CB luciferase activity in yeast cells, overnight cultures were diluted to OD_{600}=0.2 in fresh YPD medium and pregrown for 2 h at 30°C. For the 'flash lysis' method, a defined number of yeast or hyphal cells was washed and resuspended in PBS buffer (140 mM NaCl; 3 mM KCl; 8 mM NaHPO₄; 1.8 mM KH₂PO₄/pH 7.4) or water and quickly frozen in liquid nitrogen. After thawing, 100 µl of cells were placed into wells of a 96-well microtitre plate and 100 µl Chroma-Glo reagent (Promega) or luciferin reagent Beetle-glow (see following text) were added for luminescence measurements. For assays after hyphal induction the washing step was omitted. In addition to measurements by flash lysis, luciferase activity was also determined for equal amounts of viable cells, which were directly measured after washing in PBS, or after cell breakage in crude cell extracts. For preparation of crude extracts transformed cells were harvested by centrifugation (5 min; 3500 rpm) and resuspended in buffer (25 mM Tris-HCl/pH 7.5; 150 mM NaCl; 5 mM EDTA; 0.1% Triton X-100; 10% glucose) containing protease inhibitors (Cocktail Set III, EDTA-free, Calbiochem). Cells were broken by shaking with glass beads in a FastPrep homogenizer (MP Biochemicals) using six cycles of 40 s each (6.5 m/s), cooling samples on ice for 3 min after each cycle. After centrifugation for 10 min at 13 000 rpm, the supernatant (crude extract) was kept and measured.

Measurements were made in a TriStar LB 941 Luminometer (Berthold Technologies). Plates were shaken for 10–15 s at 1 m/s before the first luciferase activity measurement and for 10 s before each measurement. The reaction temperature was 30°C. For each time point, measurements were made as indicated: without optical filters; 510/50, using the green optical filter 510/50 (Chroma Technology Corporation); 610LP, using the red optical filter 610LP (Chroma Technology Corporation). For all measurements, unless otherwise specified, the exposition time was 1 s and luminescence (relative light units, RLU) was measured continuously for 20 min and the maximal luminescence value (Lmax) was reported. For simultaneous measurements of red and green luciferases, the luminescence values in each filter channel were corrected by the following equations to remove overlap signals (Davis et al., 2007):

\[
R = \frac{L_{RF} - L_{RG} \times C_{RF}}{C_{RG}} \quad G = \frac{L_{RG} - R \times C_{RG}}{C_{RF}}
\]

R' and G' represent the corrected red and green signals. Constant values (R: 14301, Rf: 7077, Rgf: 28, G: 160164, Ggf: 51230 and Gf: 6117) were determined experimentally using C. albicans transformants ACT1GU or ACT1RU. R: unfiltered luminescence of red luciferase; Rf: luminescence for red luciferase, red filter (610LP); Gf: red luciferase, green filter; G: unfiltered luminescence of green luciferase; Ggf: green luciferase, green filter (510/50); Gf: green luciferase, red filter. The lower measurement limit was set to 31 RLU (control without luciferase).

As an alternative to the commercial Chroma-Glo reagent a defined luciferin-containing substrate buffer was established according to Brasier & Fortin (2001) with several modifications (termed 'Beetleglow'). For this method, 64 µl luciferase buffer (25 mM glycylglycin/pH 7.8; 15 mM KH₂PO₄/pH 7.8; 15 mM MgSO₄; 4 mM EGTa; 2 mM ATP; 0.1% dodecyl-β-maltoside) and 36 µl luciferin solution (1 or 3 mM d-luciferin; 25 mM glycylglycin/pH 7.8) were added to 100 µl of the samples.

**HWP1 and YWP1 promoter activity during hyphal formation.** Strain YWP1GU/HWP1RH, containing a fusion of the HWP1 promoter to CaCBRluc and the YWP1 promoter to CaCBGluc, was used to determine the activity of both promoters during hyphal induction. Cells were induced to form hyphae as described above. Over a time period of 90 min 1 ml samples were taken and frozen in liquid nitrogen. Red and green luminescence were measured as described earlier using the Beetleglow substrate. Promoter activity determined for HWP1p-CaCBRluc and YWP1p-CaCBGluc fusions was normalized with measurements for strains expressing either CaCBRluc or CaCBGluc under control of the ACT1 promoter (strains ACT1RH and ACT1GU, respectively), which were grown and treated in the same way as strain YWP1GU/HWP1RH.

**qPCR.** Strain WBP17 was induced to form hyphae using 10% horse serum as described earlier; 50 ml of cells were harvested by centrifugation (3500 rpm) and frozen. Thawed cells were broken in a FastPrep homogenizer (MP Biochemicals) with 5 min cooling steps after every second cycle (six cycles; 30 s at 6.5 m/s). The samples were treated with 'RNA-clean & concentrator' (Zymo Research) and cDNA was synthesized using the 'Maxima First Strand cDNA Synthesis Kit for RT-qPCR' (Thermo Scientific). Transcript levels were measured during RT-qPCR using the 'my-Budget 5x EvaGreen™ QPCR-Mix II' (Bio-Budget) with the Mx3000P® (Stratagene) cycler using primers HWP1-L1/R1 (HWP1), YWP1 RT Fw/Bw (YWP1), CBGluc-L1/R1 (CBRLuc) and ACT1(RT)-fr (ACT1). HWP1, YWP1 and CBRLuc transcript levels were normalized to ACT1 transcript levels to determine their relative transcript levels (RTL).

**Immunoblotts.** Peptide (NH₂)SKGVVNNVEATKEADDGG, which occurs in both CBGluc and CBRLuc, was used to generate a polyclonal anti-CB luciferase antiserum in rabbit (Villalobos et al., 2010) and was provided by Genemed Synthesis Inc. For immunodetection of CaCBRluc/CaCBGluc, cells were grown to OD_{600} 0.5-0.7 and crude extracts were prepared as described above. Proteins in the crude extracts (100 µg) were separated by SDS-PAGE using a 10% polyacrylamide gel and transferred to a PVDF membrane. CaCBRluc/CaCBGluc was detected using rabbit anti-CB luciferase antiserum (1:5000), followed by treatment with anti-rabbit secondary antibody labelled with horseradish peroxidase (1: 2500) from goat. Treatment of filters with SuperSignal West Dura substrate (Pierce) generated chemiluminescent light signals, which were detected with an Image Quant LAS 400 mini imager (GE Healthcare). For determination of CaCBRluc/CaCBGluc stability (half-life) during hypha formation, ACT1RU or ACT1GU cells were induced to form hyphae using 10% horse serum, as described above, except that 1 mM cycloheximide was added at t=0 to block translation. At different time points during induction, crude extracts were prepared from 50 ml of cells and 250 µg of crude extract proteins were assayed using anti-CB luciferase antiserum by immunoblotting. CaCBGluc/CaCBRluc signals were quantified using the MultiGauge 3.0 software (FujiFilm Europe GmbH). Immunoblots were stained with Amido Black solution (0.1% Amido Black, 50% methanol, 10% acetic acid) for 5 min to monitor equal loading of the gel used for SDS-PAGE.

**RESULTS**

**Codon-optimized genes encoding CB luciferases.** Genes encoding CB luciferases have been optimized for use in mammalian cells to produce either green or red luminescence with luciferin as the substrate (Almond et al., 2003). To allow the use of CBGluc and CBRLuc in C. albicans, the corresponding genes were synthesized, in which all CUG codons were replaced by alternative leucine-encoding codons (Santos et al., 1993), the GC content was lowered and the codon usage was adapted to C. albicans. The modified CaCBGluc and CaCBRluc sequences (accession numbers KU194209 and KU194210) had a codon bias...
Fig. 1. Activity of CB luciferases in C. albicans. (a) Example for genomic integration of CaCBLuc cassette. PCR-generated integration cassettes contain CaCBGluc (CaCBrLuc) and the selection marker URA3 (HIS1) and are integrated into the target coding region by homologous recombination. Note that integration occurs in the second ACT1 exon sequence resulting in strain ACT1GU (ACT1RH). (b) Sensitivity of luciferase assay depending on cell numbers. Luciferase activity (RLU) of the indicated numbers of C. albicans cells producing CaCBrLuc (strain ACT1RU) and CaCBGluc (strain ACT1GU) was determined using the flash lysis method in the presence of commercial Chroma-Glo or the custom substrate buffer Beetleglow containing 0.5 mM D-luciferin. Dotted lines indicate background luminescence of control strain BWP17. (c) Sensitivity of luciferase assay depending on sample preparation. Transformants producing CaCBGluc were grown in YPD medium to an OD600 of 1 at 30 °C. 100 µl of viable cells in PBS or 100 µl of thawed cells in PBS after freezing in liquid nitrogen (flash lysis) or the
index of 0.9 and a GC content of 34% (formerly 0.5 and 50%, respectively). The encoded protein sequences are shown in Fig. S1, which indicate eight different residues that are responsible for the different luminescence emissions of CBGluc and CBRluc proteins.

**Luminescence of CB luciferases in C. albicans**

To verify the function of the *CaCBGluc* and *CaCBRluc* synthetic genes, they were chromosomally integrated downstream of the constitutive *ACT1* promoter in *C. albicans* strain BWP17 (Wilson *et al.*, 1999). For this purpose, PCR-generated integration cassettes comprising a *CaCBGluc* sequence, a selection marker (*URA3* or *HIS1*) and flanking sequences homologous to the target locus were chromosomally integrated by homologous recombination (scheme in Fig. 1a). Correct integration of the PCR cassette was verified in transformants ACT1GU (*CaCBGluc integration*) and ACT1RH (*CaCBRluc integration*) by colony PCR and Southern blotting (data not shown). To assay the function of both *CaCB* genes transformants were frozen in liquid nitrogen and, after thawing, were tested for green or red luminescence by addition of luciferin-containing substrate buffer (flash lysis procedure). The commercial substrate buffer Chroma-Glo (Promega) of unknown composition and the defined, custom-made buffer (named Beetleglow) were compared using different numbers of transformant cells (Fig. 1b). The results demonstrate (i) that *ACT1GU* and *ACT1RU* strains produce green or red luminescence respectively, as expected and (ii) that Chroma-Glo and Beetleglow are equally sensitive to detect about 60 cells for *CaCBGluc* and about 600 cells for *CaCBRluc*. Thus, unexpectedly, the *CaCBGluc* protein was about 10 times more sensitive than the *CaCBRluc* protein as a reporter of *ACT1* expression. Advantages of Beetleglow substrate compared to Chroma-Glo include its defined composition and lower costs. In addition, we found that Chroma-Glo but not Beetleglow blocks growth of *C. albicans*, while both substrates interfere with hyphal morphogenesis (data not shown).

For luminescence measurements, the preparation of *C. albicans* cells using the flash lysis procedure was compared with viable cells and crude cell extracts (Fig. 1c); note that equivalent amounts of cells and cell extracts were tested to make the results directly comparable. Luminescence was recorded over 90 min and the signal could be clearly detected with viable cells, although the peak of luminescence (L\text{max}) appeared only after 60 min compared to 3.5 min for flash lysis and the intensity of luminescence was about 2.5-fold lower. Most likely, the delayed L\text{max} appearance for viable cells is due to slow import of the luciferin substrate across the intact cytoplasmic membrane, as luciferin transport across plasma membranes is known to limit luciferase light output in mammalian cells (Patrick *et al.*, 2014). Using crude cell extracts, L\text{max} was rapidly reached (5 min), although its intensity was reduced compared to flash-lysed cells (2.9-fold), which is possibly due to CB luciferase degradation during cell breakage.

Collectively, the results suggest that the use of Beetleglow substrate in combination with the easy and highly sensitive flash-lysing procedure is optimal to monitor CB luciferase activity in *C. albicans*.

**CB luciferases as dual reporters of hyphal gene expression**

Hyphal morphogenesis (dimorphism) is an important virulence trait of *C. albicans* that involves numerous alterations in gene expression. Transcription of the *HWP1* gene is strongly induced during hyphal formation (Sharkey *et al.*, 1999), while *YWP1* transcription is repressed (Sohn *et al.*, 2003; Granger *et al.*, 2005). We constructed *C. albicans* strain YWP1GU/HWP1RH, in which the *CaCBGluc* coding sequence is situated downstream of the *YWP1* promoter, while *CaCBRluc* was inserted downstream of the *HWP1* promoter. Using this strain, the activity of both promoters was measured simultaneously by green and red luminescence during hypha formation. Hyphae were induced using 10% horse serum at 37°C during which a strong increase of the red *HWP1p*-dependent luminescence and a moderate increase of the green *YWP1p*-dependent luminescence were observed (Fig. 2a). To relate these luminescence values to those controlled by the promoter of a housekeeping gene, the luminescence of strains expressing *CaCBRluc* or *CaCBGluc* under control of the *ACT1* promoter (*ACT1RH, ACT1GU*) was also determined. Note that as a precaution identical selection markers (*HIS1* or *URA3*) and CB luciferase reporters (*CaCBRluc* and *CaCBGluc*) were used in these control strains. The luminescence of both control strains increased about twofold during morphogenesis, as expected for *ACT1* expression (Delbrück & Ernst, 1993). The luminescence ratio of the *HWP1RH* and *ACT1RH* strains showed a clear increase during hyphal formation, while the ratio of *YWP1GU* to *ACT1GU* luminescence was found to decrease (Fig. 2a). In parallel, using qPCR the expected increase of the *HWP1* to the *ACT1* RTL was observed, while the *YWP1* RTL was found to rapidly decrease during the first 60 min of induction (Fig. 2b). However, the relative *YWP1* transcript level decreased much more rapidly than the *YWP1p*-dependent luminescence ratio, which is presumably due to the shorter half-life of the transcript, as compared to the *CaCBRluc* protein. Furthermore, for unknown reasons, the increase of the *YWP1* transcript after 60 min of induction was not matched by an increase in the *YWP1-CaCBGluc* luminescence ratio.
Fig. 2. *HWP1/YWP1* promoter activity during hyphal formation. (a) Strain YWP1GU/HWP1RH expressing both the red (*HWP1*) and green (*YWP1*) luciferase was pre-grown overnight in YPD medium, diluted to OD600 of 0.2 in water and starved for 60 min at 30°C. At time point 0 hyphae were induced by addition of 10% horse serum and incubation at 37°C. At the indicated time points samples of the culture were assayed for red or green CB luciferase activity. In parallel, strains producing CaCBRluc (*ACT1RH*) or CaCBBGluc (*ACT1GU*) luciferase under control of the *ACT1* promoter were induced and assayed similarly. The ratio of luciferase activity in strain YWP1GU/HWP1RH to *ACT1RH* (*HWP1/ACT1* ratio) or *ACT1GU* (*YWP1/ACT1* ratio) was calculated. (b) *HWP1* and *YWP1* transcript levels. Strain BWP17 was grown and induced to form hyphae as in (a). At the indicated times of hyphal induction, total RNA was isolated and the levels of *HWP1* and *YWP1* transcript were determined by qPCR using specific primers. In addition, the *ACT1* transcript level was determined by qPCR in each sample allowing the determination of *HWP1/ACT1* and *YWP1/ACT1* ratios (RTL). Measurements and calculations are shown for two biological replicates with means and standard deviation calculated from three technical replicates.
To confirm the value of CB luciferases for dual monitoring of gene expression, we constructed strain UME6GU/EFG1RH, in which the UME6 and EFG1 promoters drive expression of CBGluc or CBRluc genes, respectively. The EFG1 transcript and promoter activity is known to decrease rapidly during hyphal induction (Tebarth et al., 2003; Lassak et al., 2011), while the UME6 transcript is rapidly upregulated (Banerjee et al., 2008). Using ACT1RH or ACT1GU control strains, luminescence ratios during hyphal induction confirmed the downregulation of EFG1 and upregulation of UME6 expression (Fig. S2), consistent with the reported transcript regulation for these genes. Collectively, the results confirm that CB luciferases are convenient tools to monitor the differential expression of two genes simultaneously in the same cell, and the results approximate transcript measurements, at best for upregulated gene expression.

**Stability of CB luciferases in C. albicans**

As a tool to monitor the presence and the stability of CB luciferases in C. albicans, a rabbit anti-CBluc antiserum was prepared. Antibody production was elicited with a peptide comprising 19 amino acids identical in CBRluc and CBGluc (marked in Fig. S1). Immunoblotting of the cell extracts with the anti-CBluc antiserum revealed the expected 60 kDa proteins in yeast-form strains ACT1RU and ACT1GU indicating that both CaCBRluc and CaCBGluc proteins are detectable by the antiserum, while only weak cross-reacting proteins of different masses were observed in the BWP17 control strain (Fig. 3a). Interestingly, using the cross-reacting bands for reference, amounts of the CaCBGluc protein appeared greater than those of the CaCBRluc protein, thus matching the higher luminescence values that were observed for this protein (Fig. 1b).

To determine the stability of both CB proteins under conditions used for hypha formation 1 mM cycloheximide was added to block protein translation during the initiation of hyphal growth in strains ACT1RU and ACT1GU. During the course of incubation, a relatively fast degradation of CaCBRluc in strain ACT1RU was observed, with an approximate half-life of 20–30 min; in contrast, the CaCBGluc protein appeared more stable with an approximate half-life of 80 min (Fig. 3b). These results suggest that the higher luminescence and protein levels observed for CBGluc (Figs 1 and 3) are due at least in part to the higher stability of the CaCBGluc compared to the CaCBRluc protein. If the half-life of YWP1p-dependent bioluminescence at about 90 min (Fig. 2a) is corrected with the CaCBGluc protein half-life of 80 min, the observed fast downregulation of the YWP1 transcript during hypha formation (5–10 min; Fig. 2b) is approximated.

**CB luciferases as reporters for inhibitors of gene expression**

*C. albicans* strains expressing CB luciferases are potentially suited to monitor inhibition of gene expression, which possibly could be useful for inhibitor screenings. To assess this notion we tested the action of farnesol, which is known to block hyphal morphogenesis in *C. albicans* (Hornby et al., 2001; Lindsay et al., 2012). Incubation of strain HWP1RH and control strain ACT1RH for 2 h under hypha-inducing conditions generated germ tube/hypha formation in >90% of cells in the absence of farnesol, while only yeast-form cells were microscopically detected in the presence of 150 μM farnesol. HWP1p-dependent to ACT1p-dependent chemoluminescence ratios were determined, which clearly demonstrated that farnesol strongly blocks the hypha-dependent upregulation of the HWP1 promoter (Fig. 4). This result fully agrees with regulation of the native HWP1 transcript and the (HWP1p-driven) CBRluc transcript, which was determined in strain HWP1RH in parallel (Fig. 4b). We conclude that *C. albicans* strains containing promoter fusions to CB luciferase genes are useful to observe the action of inhibitors individually or in mass screenings. Low background values are characteristic of luminescence measurements, allowing monitoring of subtle inhibitor activities.

**DISCUSSION**

Transcriptional circuits govern the commensal or pathogenic lifestyle of the human pathogen *C. albicans* and we report here that CB luciferases are useful tools to monitor gene expression in this fungus. Efficient production of both CBGluc and CBRluc was achieved for synthetic genes, in which all CUG codons were replaced (Santos et al., 1993), the GC content was lowered and codon usage was adapted to *C. albicans*. Importantly, both CaCBRluc and CaCBGluc luciferases yielded strong red or green bioluminescence, which could be clearly separated using selective optical filters in luminometer measurements.

To assess the application of CB luciferases, they were used to test the expression of two genes that are regulated differently during hyphal morphogenesis of *C. albicans*. While HWP1 is upregulated, YWP1 is downregulated rapidly during induction of filamentation (Sharkey et al., 1999; Sohn et al., 2003; Granger et al., 2005). The UME6 and EFG1 pair of genes is regulated similarly (Banerjee et al., 2008; Tebarth et al., 2003; Lassak et al., 2011). Promoter fusions of both genes to either CaCBluc gene in the same strain indeed demonstrated strong upregulation of red or green bioluminescence regulated by the HWP1p-CaCBrluc or, respectively, the UME6p-CaCBrluc fusion, if starved cells were induced to efficiently form hyphae by 10% serum. During serum addition, transcript levels of the ACT1 housekeeping gene are known to increase also due to resumption of growth, independently of morphogenesis (Delbrück & Ernst, 1993), which was confirmed here by luminescence measurements using control strains containing an ACT1 promoter fusion to CaCBrluc or CaCBGluc. However, morphogenesis-dependent upregulation of HWP1 (UME6) compared to growth-dependent upregulation of ACT1 was determined to be significantly greater, as shown by the high HWP1p-CaCBrluc (UME6p-CaCBrluc)/ACT1p-CaCBrluc
Click beetle luciferases in Candida

Fig. 3. Immunodetection of CB luciferases in C. albicans. (a) CaCBluc proteins during yeast growth. Cell extracts (100 µg protein) of strains BWP17 (control), ACT1GU and ACT1RU were separated by SDS-PAGE (8 % acrylamide) and blots were probed using rabbit anti-CB luciferase antiseraum (1 : 5000). The arrow indicates the migration of CaCBGluc and CaCBluc proteins. (b) Stability of CaCBluc and CaCBGluc during hyphal induction. Strains producing CaCBluc (ACT1RU) or CaCBGluc (ACT1GU) under control of the ACT1 promoter were induced to form hyphae using 10 % horse serum as described in Fig. 2, except that 1 mM cycloheximide was added to block translation. At the indicated time points crude cell extracts (250 µg protein) were separated by SDS-PAGE (10 % acrylamide) and immunoblotted as in (a). The CaCBluc band on the immunoblots was quantitated by scanning and expressed as a percentage of the t=0 min value. An unknown protein detected by Amido black staining of the immunoblots is shown as the loading control for SDS-PAGE.

ratio, which approximated the relative HWP1/ACT1 level of the native transcripts measured by qPCR. Thus, relative bioluminescence, which is easily determined in whole or flash-lysed cells, generated results on gene expression that resembled laborious measurements of relative transcript levels. As for genes downregulated during morphogenesis, relative bioluminescence ratios for YWP1 and EFG1 promoter fusions were shown to decline, as expected. However, while the native YWP1 transcript level initially decreased rapidly during hyphal induction, only a moderate downregulation of the relative YWP1 promoter-mediated CaCBluc bioluminescence was observed. Similarly, the reported rapid decline of the EFG1 transcript during morphogenesis (Tebarth et al., 2003; Lassak et al., 2011) was not matched quantitatively by the reduction of the EFG1 promoter-dependent relative bioluminescence. These results suggested that bioluminescence measurements of downregulated genes were obscured by the stability of CB proteins. Although both CaCBluc and CaCBluc differ in only eight amino acids (Almond et al., 2003), both proteins differed significantly with regard to protein stability. Using immunoblotting with a newly generated anti-CB luciferase antibody the half-life of CaCBluc was estimated as 20–30 min, while about 80 min was found for CaCBGluc. Thus, the higher light output of CaCBluc as compared to CaCBluc may at least in part be due to the observed higher stability of this luciferase compared to CaCBluc. Because of its shorter half-life, CaCBluc may be preferred to CaCBGluc to monitor downregulation of promoter activity, provided that its bioluminescence output suffices. At late times of hyphal induction (>60 min) we detected another discrepancy between the relative transcript and luminescence values for the YWP1 gene, because at this time its transcript level rose, while the luminescence remained constant. We speculate that at this time point the stability of the YWP1 transcript begins to increase and/or that translation of the YWP1p-CBGluc transcript is halted. We note that a similar U-shaped pattern of transcript occurrence has been observed previously for transcripts of yeast-specific regulators including EFG1 and NRG1 (Tebarth et al., 2003;
Lassak et al., 2011; Lu et al., 2011), a pattern which at late induction times may support lateral budding of yeast cells from hyphae.

Unlike RLUC and GLUC luciferases, bioluminescence by CB luciferases is ATP-dependent. This property is of special importance for non-invasive real-time monitoring of host–pathogen interactions in animals, since only live, and not dead, fungi will be visualized. On the other hand, their ATP dependence precludes the activity of CB luciferases on the fungal cell surface, as has been done for the GLUC luciferase to ease in vivo availability of its coelenterazine substrate (Enjalbert et al., 2009). Dual bioluminescence output has been described previously for a commercial system (Dual-Light, Applied Biosystems), in which two bioluminescent substrates of β-galactosidase and FLUC are added sequentially to cell extracts. However, the use of a single substrate for two gene activities and the possibility of even using live cells make CB luciferases the preferred tools for monitoring gene expression in the future. The possibility to rapidly monitor the expression of two genes simultaneously (e.g. a target and a reference gene) using the same fluorescent substrate (luciferin), may make CB luciferases especially important for mass screenings to identify novel inhibitors of C. albicans gene activities, which may be developed into novel antifungal compounds.

**Fig. 4.** Inhibition of *HWP1* promoter activity by farnesol. Strain HWP1RH producing CaCBRluc under transcriptional control of the *HWP1* promoter, as well as control strain ACT1RH were induced to form hyphae for 2 h using 10% serum as described in Fig. 2, except that some cultures received 150 µM farnesol to block hypha formation. (a) Luminescence of strains HWP1RH and ACT1RH; (b) *HWP1* and *CBRluc* transcript levels of strain HWP1RH. Relative *HWP1* luminescence and relative *HWP1* or *CBRluc* transcript levels were calculated for three technical replicates as in Fig. 2.
Here we have obtained the first evidence for this application by demonstrating the inhibitor activity of farnesol, which previously has been described to block hypha formation and hypha-dependent gene expression (Hornby et al., 2001; Lindsay et al., 2012). Conversely, not only inhibitors of hypha formation but also inducers of the yeast form of *C. albicans* may be identified by mass screenings in molecular libraries.

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