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

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Synthetic genes encoding functional luciferases of the click beetle (CB) *Pyrophorus plagiopthalamus* have been expressed in the human fungal pathogen *Candida albicans*. Both green- and red-emitting CB luciferases (CaCBGluc and CaCBRluc) were produced with high efficiency in transformants under transcriptional control of the growth-dependent ACT1 promoter, as well as by the YWP1 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 CaCBRluc and 80 min for CaCBGluc, as determined by immunoblotting). Importantly, the activity of both luciferases could be separately 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 *YWP1p-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 plagiopthalamus* 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 *CBLuc* and *CBGluc* genes encoding luciferases producing well-separated red and

**Abbreviations:** ATP, adenosine triphosphate; Ca, *Candida albicans*; CB, click beetle; FLUC, firefly luciferase; GLUC, *Gaussia princeps* luciferase; L<sub>max</sub>, 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 strain (ELuc9 from Pyrearinus termittiliuminus) also was found to be superior to FLUC (Nakajima et al., 2010), and its combination with red-emitting Stable Luciferase 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 resuspension in sterile water at an OD

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 Ura3' (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 HWPIRH. In this case, the insertion cassette was amplified by PCR using primers inHWPIRH Fw/Bw and plasmid pGEM-HIS-CBR as template; correct chromosomal insertion was verified by colony PCR using primers HWPI Dia Fw/Luci Dia Bw generating a 716 bp fragment and subsequent Southern blotting (data not shown). To place *CaCBGluc* downstream of the *HWP1* promoter the insertion fragment was generated by PCR using primers inHWPIGU Fw/Bw and plasmid pD-CBG as the template; correct chromosomal insertion was verified by colony hybridization using primers YWPI Dia Fw/Luci Dia Bw generating a 1273 bp fragment and subsequent Southern blotting (data not shown). The resulting verified transformant was named YWPIGU, strain YWPI1GU/HWPIRH contains the fusion of the *HWP1* promoter to *CaCBRluc*, which was transformed in strain YWPIGU. 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

<table>
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<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>BWP17</td>
<td>ura3-iro1A::him434/ura3-iro1A::Amn434 hisG/his1::hisG arg4::hisG arg4::hisG</td>
<td>Wilson et al. (1999)</td>
</tr>
<tr>
<td>ACT1GU</td>
<td>as BWP17 but ACT1/act1::(ACT1p-CaCBGluc URA3)</td>
<td>This work</td>
</tr>
<tr>
<td>ACT1RH</td>
<td>as BWP17 but ACT1/act1::(ACT1p-CaCBRluc HIS1)</td>
<td>This work</td>
</tr>
<tr>
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<td>as BWP17 but ACT1/act1::(ACT1p-CaCBRluc URA3)</td>
<td>This work</td>
</tr>
<tr>
<td>YWPI1GU</td>
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<td>This work</td>
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<td>This work</td>
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<td>This work</td>
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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 (1099 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<sub>600</sub>=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 Na<sub>2</sub>HPO<sub>4</sub>; 1.8 mM KH<sub>2</sub>PO<sub>4</sub>/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 microtiter 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 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 (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 Lumimeter (Berthold Technologies). Plates were shaken for 10–15 s at 1 m/s before the first luciferase activity measurement and for 1 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 (L<sub>max</sub>) 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):

\[
R = \frac{L_{gf} - L_{gf}\times \frac{Grf}{Grf}}{R - \frac{G}{Gf}}
\]

\[
G = \frac{L_{gf} - R \times \frac{G}{Gf}}{R - \frac{Grf}{Grf}}
\]

\(R\) and \(G\) represent the corrected red and green signals. Constant values (R: 14301, Rf: 7077, Rgf: 28, G: 160164, Gf: 51230 and Grf: 6117) were determined experimentally using \(\text{Maxima First Strand cDNA Synthesis Kit for RT-qPCR} (\text{Thermo Scientific})\). Transcript levels were measured during RT-qPCR using the ‘my-Budget 5x EvaGreen™ QPCR-Mix II’ (Bio-Budget) with the Mx3000P® (Stratagene) cycling using primers HWPI1-L1/R1 (HWPI1), YWP1 RT Fw/Bw (YWP1), CBBluc-L1/R1 (CBBLUC) and ACT1(RT)-rt (ACT1). HWPI1, YWP1 and CBBLUC transcript levels were normalized to ACT1 transcript levels to determine their relative transcript levels (RTL).

**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 \(\text{CBGluc} \) and \(\text{CBBruc}\) in \(\text{C. albicans}\), 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 \(\text{C. albicans}\).

The modified \(\text{CaCBGluc} \) and \(\text{CaCBBruc}\) 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 *OD*<sub>600</sub> 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
equivalent amount of crude cell extract were compared for CB luciferase activity using Beetleglow containing 0.5 mM D-luciferin (luminescence measurements for 90 min without optical filter).

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 CaCBGluc 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 but Chroma-Glo and Beetleglow (Promega) 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 luciferase 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 (HWP1p) and green (YWP1p) luciferase was pre-grown overnight in YPD medium, diluted to \( \text{OD}_{600} \) 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 CaCBGluc (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 CBLuc proteins for dual monitoring of gene expression, we constructed strain UME6GU/EFG1RH, in which the UME6 and EFG1 promoters drive expression of CBGluc or CBBrLuc genes, respectively. The EFG1 transcript and promoter activity is known to decrease rapidly during hyphal induction (Tebart 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 lucerase 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 luciferses, 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 the 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
ratio, which approximated the relative $HWPI/ACT1$ 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 $YWPI$ and $EFG1$ promoter fusions were shown to decline, as expected. However, while the native $YWPI$ transcript level initially decreased rapidly during hyphal induction, only a moderate downregulation of the relative $YWPI$ promoter-mediated CaCBGluc 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 CaCBGluc and CaCBRluc 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 CaCBRluc was estimated as 20–30 min, while about 80 min was found for CaCBGluc. Thus, the higher light output of CaCBGluc as compared to CaCBRluc may at least in part be due to the observed higher stability of this luciferase compared to CaCBRluc. Because of its shorter half-life, CaCBRluc 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 $YWPI$ 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 $YWPI$ transcript begins to increase and/or that translation of the $YWPIp-CaCBGluc$ 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;
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 molecule libraries.

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